Endosomal Maturation Is Controlled By A Rab7 Feedback Loop Concomitant With Vesicular Interactions

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Summary

Endosomal maturation controls trafficking and degradation of macromolecules in eukaryotic cells and represents an important regulatory point in the endocytic pathway. Progression through this phase is primarily recognized through the change of endocytic membrane proteins, particularly the transition from a Rab5 to Rab7 positive compartment. This specific detachment/attachment of Rab5 and Rab7 respectively, seems to regulate this checkpoint in endo-lysosmal pathway via a cascade of complex interactions, emphasizing mutual feedback mechanisms. The Rab proteins have over the last two decades been connected to several important roles in various vesicular trafficking pathways in the cell. While the process of maturation is a complex event, involving many different effector proteins, there is a growing understanding of what roles the different proteins inhabit and the dynamic interplay between them.

In this project we set out to establish a method for investigating if Rab7 has a regulatory role on the coat dynamics of Rab5. We made use of three different inducible cellular systems that have been known to generate enlarged endocytic compartments and transiently transfected these cells with Rab5-wt-mCherry, Rab7-wt-EGFP, Rab7-Q67L-EGFP or Rab7-T22N-EGFP. From this enlarged endocytic structures co-transfected with Rab5-wt-mCherry and Rab7 alternates we were able to accurately measure the kinetics of Rab5-wt-mCherry. The binding dynamics of Rab5-wt-mCherry were measured prior to and during maturation. Moreover, Rab5-mCherry coat detachment during maturation was quantified by calculating the maturation half time (M½) and the maturation end point (M_{end}) as a function of Rab5-wt-mCherry (see materials and methods). Prior to maturation we measured the on/off-cycling of Rab5-wt-mCherry by FRAP experiments on stable early endosomes and calculated the T½ recovery of Rab5-wt-mCherry. In cells expressing either Rab7-wt-EGFP or the mutants, any perturbations in the Rab5-wt-mCherry coat kinetics prior to and during maturation may imply a regulatory role for Rab7 in Rab5 coat kinetics.

Our results indicate that Rab7 is likely to play an important role in controlling the kinetics of Rab5. The different Rab7 mutants that were transiently transfected into the various systems led to some interesting results in Rab5 coat dynamics, both during and prior to maturation.

Expressing the dominant active Rab7-Q67L-EGFP led to an increase in T½ recovery on stable early endosomes, it led to a decrease in the time of maturation. While the dominant negative mutant Rab7-T22N-EGFP had no effect on on-off kinetics Rab5 on stable early endosomes during the FRAP experiments. However, the coat dynamics of Rab5-wt-mCherry was highly unstable in cells expressing Rab7-T22N-EGFP. This was observed as a reduced number of early endosomes maturing, while those maturing displayed an unusually fast Rab5 coat detachment.

Furthermore, we observed an increased amount of vesicular interactions around the maturing endosome. We believe that this, together with the Rab7 positive feedback loop, represents an important regulatory factor during the endosomal maturation.

Abbreviations

AP	adaptor protein
APPL	Adaptor protein containing PH domain, PTB domain and leucine zipper
ATP	Adenosine triphosphate
CCP	Clathrin coated pits
CCV	Clathrin coated vesicle
CME	Clathrin mediated endocytosis
CORVET	Vacuole and a novel endosomal tethering complex
DRM	Detergent-resistant membranes
EE	Early Endosome
EEA1	Early Endosomal Antigen1
EGF	Epidermal Growth factor
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal sorting complex required for transport
FRAP	Fluorescent recovery after photobleaching
FYCO1	FYVE and coiled coil domain containing 1
FYVE	PtdIns(3)P binding domain
GAP	GTPase activating proteins
GDF	GDI-displacement factor
GDI	GDP-dissociation inhibitor
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HOPS	homotypic fusion and vacuole protein sorting complex
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
Ii	Invariant chain
IL-2R-β	Interleukin-2β-chain receptor
ILEV	Invariant chain enlarged vesicle
ILV	Intraluminal vesicles
LAMP	Lysosome-associated membrane protein
Le	Late Endosome
LIMP	Lysosome integral membrane protein
M6PR	Mannose-6-Phosphate receptor
MDCK	Madin Darly canine kidney cells
MVBs	Multivesicular Bodies
NSF	N-ethylmaleimide-sensitive factors
ORP1L	(oxysterol-binding protein) related protein
PM	Plasma membrane
PtdIns	Phosphoinositide
PX	Phox-homology
RFP	Red fluorescent protein
RI	Relative intensity
RILP	Rab7 interacting lysosomal protein
ROI	Region of interest
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment receptor proteins
SNX	Sorting nexins
TfR	Transferrin
TGN	Trans Golgi Network
TIP47	Tail-interacting protein 47
UVRAG	UV Radiation resistance associated gene
VPS	Vacuolar protein sorting
WT	Wild Type

1. General Introduction

1.1. Vesicular Pathways Of The Cell.

1.1.1. Endocytosis

Endocytosis is the process in which cells absorb nutrients by engulfing them in vesicles created by invaginations of the plasma membrane (PM). Over the course of evolution several distinct varieties have emerged, and the cell relies on these to acquire macromolecules and extracellular fluids needed for both growth and stability. The cargo can be newly absorbed molecules which are to be recycled or it can be molecules needed for cell to cell communication, PM composition, and immune response related events. Simply described one can say that the macromolecules are sorted into invaginations at the PM, which then are pinched off into a vesicle and released into the cytosol of the cell. The destiny of the vesicle depends on the cargo, but it will likely follow the degradative pathway to the lysosome where the content will be degraded before it is released to the cytosol for further use of the cell. In contrast to endocytosis, exocytosis is the transport of cargo from the cell to the extracellular environment and is important in PM composition and in situations such as an immune response.

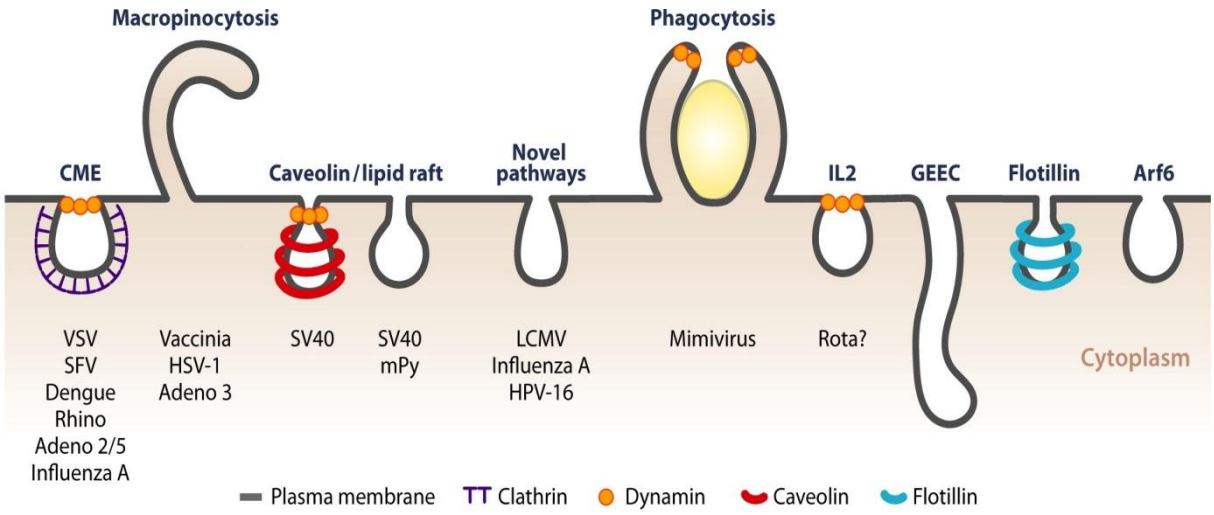

FIGURE 1.1-1-1: **THE MANY MECHANISMS OF ENDOCYTOSIS**. HERE ONE CAN SEE MOST OF THE MECHANISMS UTILIZED BY A CELL TO INTERNALIZE FLUIDS OR MACROMOLECULES FROM THE EXTERIOR OF THE CELL. THE ENDOCYTIC PATHWAY IS USUALLY DECIDED DEPENDENT ON THE CARGO INTERNALIZED AND MOST OF THE DIFFERENT PATHWAYS HAVE BEEN DISCOVERED DUE TO THE OBSERVATION THAT THEY SERVE AS ENTRY POINT FOR VARIOUS VIRUSES (MERCER, SCHELHAAS ET AL. 2010).

Over the years, several distinct ways of endocytosis have been discovered (Figure 1-1). The most fully understood system of endocytosis, the Clathrin-dependent pathway or Clathrin mediated endocytosis (CME), is also the endocytic process of which most extracellular content is internalized (Sorkin 2004). Clathrin also has other roles in the cell beside endocytosis. It is also associated with vesicle traffic from the trans golgi network (TGN), shipping macromolecules to the plasma membrane or to the exterior of the cell. CME has a large number of effector proteins which secure cargo specificity, membrane curvature, and membrane scission and more, however, it is not unlikely that these other mechanisms of endocytosis will grow in importance as they receive increased attention comparable to CME. Besides CME, there is the debated caveolar-dependent endocytosis (Rothberg, Heuser et al. 1992), macropinocytosis (Damke, Baba et al. 1995), the Cdc42-dependent pathway (Sabharanjak, Sharma et al. 2002), the RhoA/Rac1 dependent pathway (Lamaze, Dujeancourt et al. 2001), the Arf6-dependent endocytosis (Naslavsky, Weigert et al. 2004), and the Flotillin-dependent endocytosis (Glebov, Bright et al. 2006).

Clathrin coated pits (CCP) formation is the initial step in CME where invaginations occur in specialized areas in the PM that are enriched with phosphoinositides (PtdIns) such as PtdIns(4,5)P2 and PtdIns(3,4,5)P3. These areas serve as binding sites for the assembly of a complex consisting of Adaptor Protein (AP) 2 (Gaidarov, Chen et al. 1996) and AP180 (Ford, Pearse et al. 2001). These are required for the recruitment of Clathrin to the membrane through interactions with the N-terminal region of the Clathrin heavy chain (Shih, Gallusser et al. 1995; Gaidarov, Chen et al. 1996). The Clathrin is recruited to the CCP as a triskelion structure, forming a distinct cage like structure around the Clathrin coated vesicle (CCV). AP-2 also plays an important role in the sorting of cargo into CCPs (Schmidt, Briese et al. 2006).

Additional adaptor proteins that interact in this process are: Epsin which is required for AP-2 function and promotes membrane curvature (Ford, Mills et al. 2002). Epsin, dynamin, Rab5 and bar domain-containing protein are found in roles that participate in the process of invagination and sorting into CCP (McLauchlan, Newell et al. 1998; Sweitzer and Hinshaw 1998; Ford, Mills et al. 2002). Eps15 can aid in the clustering of AP-2 in the CCPs, indirectly promoting increased concentration of cargo by increasing AP-2 ability to interact with other

effectors (Schmid, Ford et al. 2006). The scission from the membrane is dynamin dependent (Oh, McIntosh et al. 1998) and endophilin and actin filaments contribute in the formation of CCV. The Clathrin coat rapidly disassociates from the CCV after release from the plasma membrane by the paired action of Hsc70 and auxilin (Barouch, Prasad et al. 1997; Rothnie, Clarke et al. 2011), and picks up the characteristics of an early endosome.

The Caveolin-dependent pathway's role as a starting point in vesicular trafficking of the cell is disputed (Parton, Joggerst et al. 1994; Thomsen, Roepstorff et al. 2002). It is recognized by the distinct flask like invaginations ranging from 50-80 nm coated by Caveolin-1 and Cavins (Rothberg, Heuser et al. 1992). The pathway is believed to have a role in the endocytosis of ligands such as albumin, autocrine motility factor, tetanus toxin, cholera toxin and certain viruses (Tiruppathi, Song et al. 1997; Pelkmans, Kartenbeck et al. 2001; Trushina, Singh et al. 2006). However it seems more apparent that the unique flask like buds at the plasma membrane are static (Thomsen, Roepstorff et al. 2002) and not internalized into the recently extinct concept of caveosomes (Hayer, Stoeber et al. 2010). The cargo that was recently believed to be internalized by this pathway is now thought to be incorporated into more conventional endocytic routes. Furthermore, the caveolae might play a role in trans endothelial transport or as a regulatory role in other endocytic pathways (Simionescu, Gafencu et al. 2002; Sandvig, Pust et al. 2011).

The RhoA/rac1 endocytic pathway is most likely a Dynamin dependent route into the cell discovered in context of its role in the uptake of the interleukin-2β-chain receptor (IL-2R-β) (Lamaze, Dujeancourt et al. 2001). It is also suggested to be involved in the uptake of other proteins implicated in immunology, which are clustered into detergent-resistant membranes (DRMs). Furthermore, in contrast to the CCVs, the cargo is released into the cell in uncoated vesicles (Sauvonnet, Dujeancourt et al. 2005; Fattakhova, Masilamani et al. 2006).

The CDC42 endocytic pathway is a cholesterol dependent- and Dynamin-independent pathway. It is responsible for a significant fraction of fluids in the cell, is also responsible for the uptake of a variety of viruses and GPI-anchored proteins that are concentrated in DRMs (Sabharanjak, Sharma et al. 2002; Chadda, Howes et al. 2007). The cargo is concentrated into distinct endosomes termed GPI-AP-enriched early endosomal compartments.

1.1.2. Cellular Vesicle Trafficking

The transport of ligands, receptors and lipids between the compartments of the cell is mostly achieved by specific vesicular transport. Endocytosed cargo is usually sent along the path of the endo-lysosomal pathway (Figure 1-2), defined as a route for endocytosed cargo to the lysosome for degradation, that undergoes several distinctive steps along the way. The first of these are the compartment known as the early endosome (EE). Newly formed EEs originating from endocytosis undergo homotypic fusion resulting in larger EEs with a more specialized role in recycling and sorting. This EE is often referred to as a sorting endosome, but gradually changes, attaining a more spherical morphology. The next step is when the EE matures into late endosome (LE). In LEs the formation of intraluminal vesicles (ILVs) to make *trans* membrane receptor proteins more easily accessible for degradation after the LE fuse with a lysosome.

The pH of the different endosomal compartments vary (Maxfield and Yamashiro 1987). Starting of as slightly acidic in the EEs; it decreases to become more and more acidic before ending at the lowest point found in lysosomes. This is useful as many biological processes performed by their individual endosomal compartments have their own preference to the pH of the environment they work in. Responsible for this continuous acidification is the ATP-dependent proton pump known as the V-ATPases located to the membrane of the endosomal organelles. Processes, which require a certain acidic environment, include intracellular transport, fusion and immune responses. A low endosomal pH is for instance necessary for endosomes to interact with Arf1 dependent COP-positive vesicles (Gu and Gruenberg 2000). The proper interactions required for the formation of the *trans*-SNARE complex during docking and fusion is also dependent on the proper pH (Ungermann, Wickner et al. 1999).

A diverse number of signaling markers and effector proteins are utilized to achieve the exact and precise coordination of cargo around the cell. Examples of such proteins are the COP-proteins, Sorting nexins (SNXs), SNAREs, Rabs, PtdIns and APs. They secure that budding, sorting, fission, motility, tethering and fusion are achieved at the right time and place. These events are essential for the cellular machinery to work in an effective manner.

The vesicle transport is coordinated along the cytoskeletal network. The cytoskeleton is built up by microtubules, intermediate filaments and actin filaments, where only microtubules and actin are involved in vesicular trafficking. Microtubules and actin do this by recruiting motor proteins such as kinesins (Olmsted 1986; Liao and Gundersen 1998), myosins and dyneins. Most vesicles are able to recruit a variety of effector proteins to achieve contact with a certain motor protein, allowing vesicular trafficking to be a highly dynamic process with high specificity, essential for the viability and functionality of a cell. Kinesins and dynein motor proteins are for instance involved in activities such as fission of EEs and sorting of cargo into degradative and recycling compartments (Bananis, Murray et al. 2000; Hoepfner, Severin et al. 2005; Driskell, Mironov et al. 2007) and that they also contribute to homotypic fusion events between endosomes in different parts of the degradative pathway (Bomsel, Parton et al. 1990; Aniento, Emans et al. 1993; Driskell, Mironov et al. 2007). Depolymerization of the microtubules results in an altered distribution of LEs and lysosomes in the cytoplasm, interference with maturation, an increased Rab5 and Rab7 co-localization and a dispersal of EEs throughout the cell (Bayer, Schober et al. 1998). The similar effects have been proven by inhibition of the motor protein Dynein (Driskell, Mironov et al. 2007).

Kinesins are mainly associated with the plus-end-directed movement along microtubules and is through different combinations of effector proteins able to connect to EEs and LEs (Brown, Maier et al. 2005; Hoepfner, Severin et al. 2005; Hirokawa, Noda et al. 2009). Dynein on the other hand is thought to be responsible for the majority of minus-end-directed transport (Aniento, Emans et al. 1993; Bananis, Nath et al. 2004). Vesicles bind to dynein in either a direct or indirect manner. In the direct binding Dynein binds via intermediate, light-intermediate or light chains of dynein. In the indirect manner, dynein binds via an adaptor complex (Progida, Malerod et al. 2007). LEs for instance connect to dynein through the dynactin multi protein complex and the LE coat protein Rab7 (Jordens, Fernandez-Borja et al. 2001).

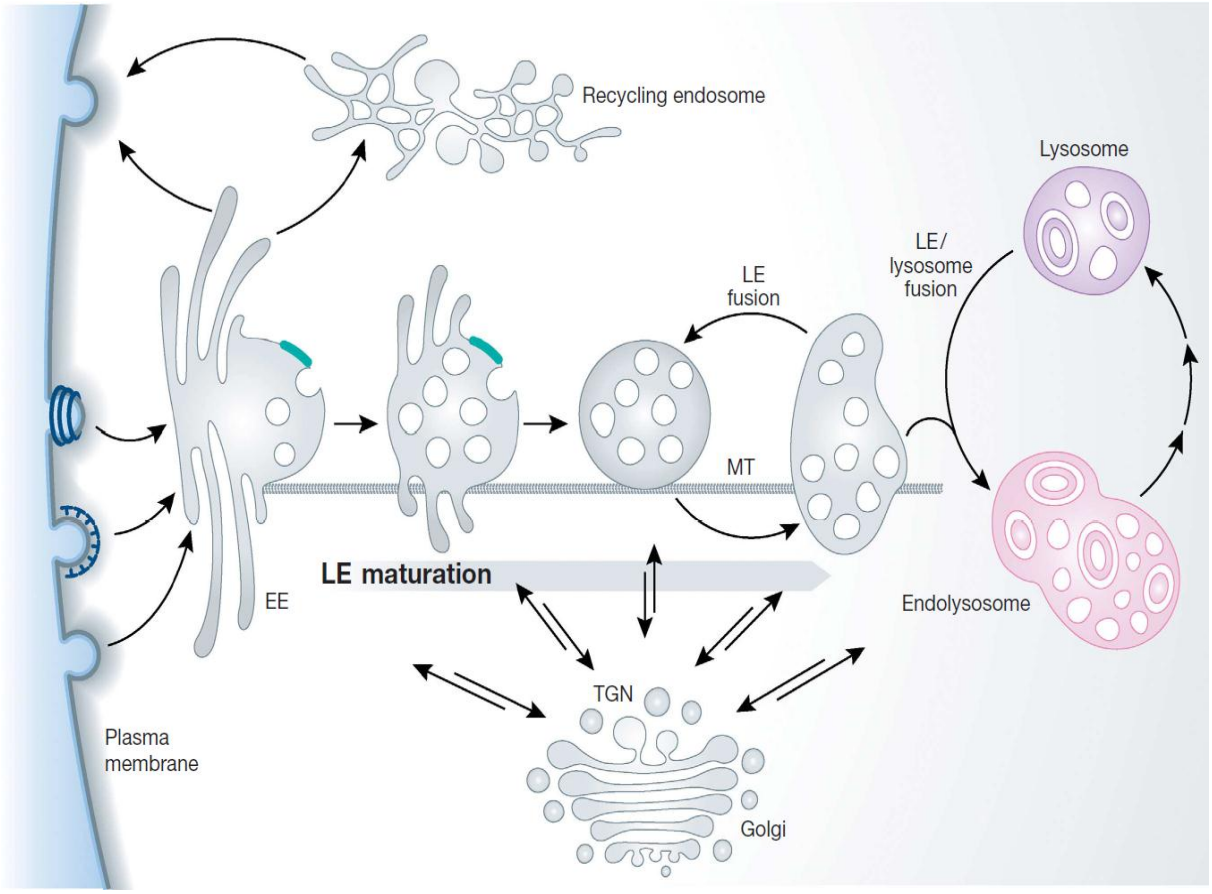

FIGURE 1-2: **THE ENDOSOMAL PATHWAY**. ENDOCYTOSED MATERIAL IS MOST LIKELY TO CONVERGE AT THE POINT OF EARLY ENDOSOMES. THIS EARLY ENDOCYTIC COMPARTMENT HAVE A SIGNIFICANT ROLE IN THE SORTING OF CONTENT. MUCH OF THE SORTING SPECIFICITY IS ACHIEVED BY TUBULAR EXTENSIONS WITH DISTINCT MICRODOMAINS OF COAT PROTEINS.THESE DECIDE IF CARGO IS TO BE RECYCLED DIRECTLY TO THE PLASMA MEMBRANE OR IF IT IS TO BE RECYCLED INDIRECTLY THROUGH A TUBULAR RECYCLING ENDOSOME. THE TUBULAR EXTENSIONS ARE LOST AS THE EARLY ENDOSOME GRUADUALE MOVE ALONG THE ENDOSCYTIC PATHWAY AND THE MORPHOLOGY BECOMES MORE SPHERICAL AND INTRALUMINAL VESICLES BECOME PRESENT IN THE LUMEN OF THE ORGANELLE. THE EARLY ENDOSOME MATURES INTO A LATE ENDOSOME IN A SERIES OF COAT PROTEIN REPLACEMENTS. THE FORMATION OF INTRALUMINAL VESICLES ARE INCREASED. THE LATE ENDOSOMES FUSE WIT LYSOSOMIC COMPARTMENTS TO CREATE A HYBRID ORGANELLE, IN THE FIGURE NAMED ENDOLYSOSOME, RELEASING ITS CONTENT FOR DEGRADATION BY THE ACID HYDROLASES PRESENT. ALL ALONG THIS PATHWAY THERE IS INTERACTIONS WITH THE TRANS GOLGI NETWORK AND THERE IS ALSO A STEADILY DECREASE IN THE LUMEN PH. ((HUOTARI AND HELENIUS 2011).

1.2. Rab GTPases And Other Proteins Involved In Vesicular Trafficking

1.2.1. Rab GTPases

Vesicular trafficking is controlled by a variety of different helper proteins. This includes the Rab proteins, which are small GTPases that are members of the Ras-superfamily. To date there are over 70 members of the Rab family that have been categorized, and their composition on membranes ensures organelle identity and function.

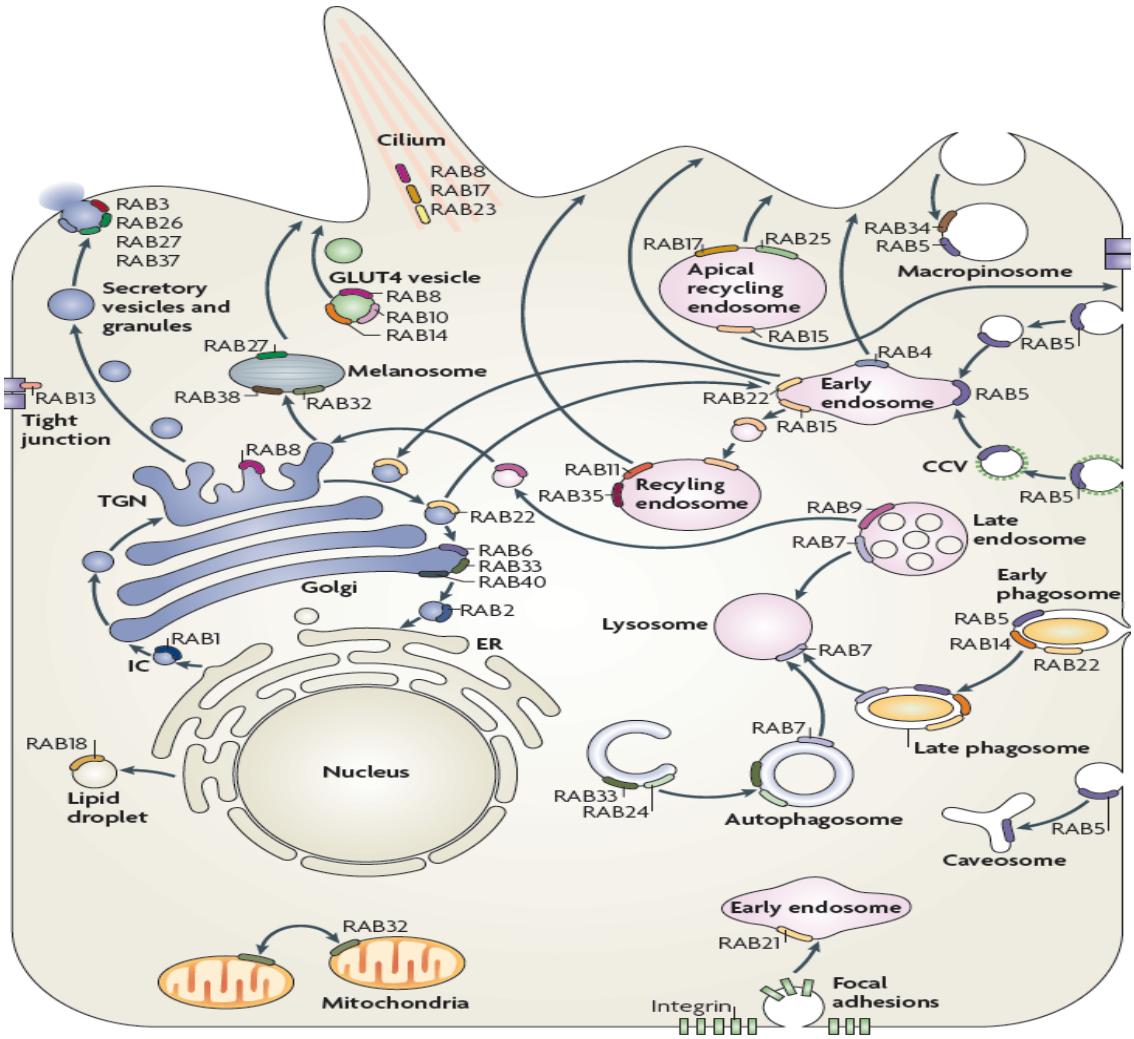

FIGURE 1-1.2-1: **THE MANY ROLES OF RAB GTPASES**. THE RAB PROTEINS ARE PRESENT AT MOST, IF NOT ALL, LIMITING MEMBRANES THROUGHOUT THE CELL. THEY SERVE IN A MULTITUDE OF ROLES RANGING FROM TETHERING, BUDDING, MOTILITY AND RECRUITERS FOR OTHER EFFECTOR PROTEINS THAT ARE NEEDED FOR SPECIFIC ORGANELLE INTERACTIONS. THE IMPORTANCE OF MANY OF THESE RAB PROTEINS HAS BEEN SHOWN AS MANE SEVERE IMMUNOLOGICAL DEFECTS HAVE BEEN ATTRIBUTED TO DEFECTIVE RABS (Stenmark 2009).

Rab proteins are able to perform multiple roles ranging from transport, sorting, budding, tethering, fusion and motility by cycling between the active GTP-bound state and the inactive GDP-bound state (Soldati, Shapiro et al. 1994; Ullrich, Horiuchi et al. 1994). In the cytosol, prenylated Rabs can be found bound to a GDP-dissociation inhibitor (GDI) that covers up the prenyl groups (Shapiro and Pfeffer 1995). In order for a Rab to switch to the active GTP-bound state, the GDI has to be displaced, and this is done by the action of a GDI-displacement factor (GDF) (Sivars, Aivazian et al. 2003). The GDFs are found on the membrane of organelles and aid Rabs specific for the membrane to remove GDI allowing the Rab GTPase to attach to the determined membrane, that will in turn activate them by GDP/GTP exchange catalyzed by a Guanine exchange factor (GEF) (Dirac-Svejstrup, Sumizawa et al. 1997; Sivars, Aivazian et al. 2003). Even though the Rabs have intrinsic hydrolytic activity towards GTP, the process of inactivation is catalyzed by GTPase activating proteins (GAPs); resulting in a renewed association with the GDI, recycling the Rab GTPases back to the cytosol.

The Rab GTPases have roles in budding, uncoating, motility and tethering by recruiting effector proteins needed to resolve these mechanisms. In the process of budding GTPases like rab9 and rab5 play active roles in concentrating cargo in emerging vesicles (McLauchlan, Newell et al. 1998; Carroll, Hanna et al. 2001). Overexpression of Rab5wt was found to result in a maximized internalization of Transferrin (TfR), while GDP-locked Rab5 inhibited the uptake (Bucci, Parton et al. 1992). Later it was reported that Rab5-GDI is an essential factor for sequestration of TfR into CCPs (McLauchlan, Newell et al. 1998). Rab9 on the other hand has been shown to recruit a protein named Tail-interacting protein (TIP47) to LEs (Carroll, Hanna et al. 2001). TIP47 is essential for the recycling of the Mannose-6-Phosphate receptor **(**M6PR**)** back to TGN. By binding to Rab9 it is efficiently targeted to the membranes of LEs, while at the same time increasing TIP47 binding capabilities to M6PR (Carroll, Hanna et al. 2001).

Rab5 is also involved in the recruitment of Early Endosomal Antigen-1 (EEA1), an effector protein that is exclusively found on EEs that are involved in the homotypic fusion process (Simonsen, Lippe et al. 1998). After EEA1 is located to the membrane of EEs it is able to directly interact with syntaxin-6 and syntaxin-13, effector proteins essential for fusion

(Christoforidis, McBride et al. 1999; McBride, Rybin et al. 1999; Simonsen, Gaullier et al. 1999). EEA1 is therefore essential for docking and homotypic fusion of EEs.

Furthermore, a different role for Rab5 has been implicated in uncoating of CCVs. Rab5 in combination with its GEFs, hRME-6 and rabex-5(Sato, Sato et al. 2005), is required for the efficient release of AP-2 from CCVs (Semerdjieva, Shortt et al. 2008). Rab5 and gRME-6 induce this by promoting dephosphorylation of the AP-2 subunit µ2. This is likely to be a direct consequence of an immediate displacement of the AP-2 associated kinase 1 (Conner and Schmid 2002), which role is phosphorylation of µ2 (Semerdjieva, Shortt et al. 2008). An increased turnover of PI(4,5)P_2 occurs as well, likely due to recruitment of a class I PI 3-kinase promoted by Rab5 (Semerdjieva, Shortt et al. 2008).

In addition to tethering and fusion, Rab5 is also involved in motility. Rab5 stimulates minus-end-directed movement along microtubules (Nielsen, Severin et al. 1999), probably quite similar to the control Rab6 have in the retrograde transport from Golgi to ER (Echard, Jollivet et al. 1998; White, Johannes et al. 1999). Other examples of motility regulated by Rab GTPases are Rab27a, which regulates the distribution and movement of melanosomes (Kuroda and Fukuda 2004). Rab7 is able to interact with dynein-dynactin motor complexes through the recruitment of an effector protein named Rab7 interacting lysosomal protein (RILP) that moves Rab7 positive compartments toward the perinuclear region of the cell (Jordens, Fernandez-Borja et al. 2001).

1.2.2. Phosphoinositides: Membrane Specific Lipid Markers.

It is not only the Rab GTPases that ensure membrane identity. A small group of specialized lipids called Phosphoinositides (PtdIns(x)P) can be found along the routes of vesicular trafficking. Assigned to a few or only one organelle these lipids provide an identity marker to which other effector proteins can be explicitly recruited.

PtdIns(x)P emerge when their elementary arrangement, the phosphatidylinositols (PtdIns), is phosphorylated at hydroxyl groups 3,4,5 in the inositol ring (Figure 4). Through their specific interactions with PI kinases and phosphatases they exert their role

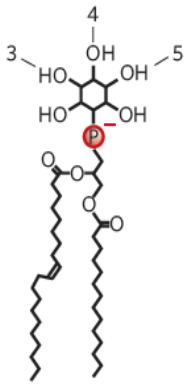

FIGURE 1-1.2-2: **BASIC STRUCTURE OF A PHOSPHATIDYLINOSTIOL** (Behnia and Munro 2005)

in asserting membrane identity, trafficking and recruitment of effector proteins. EEs are for instance highly enriched in PtdIns(3)P, which is generated by a class III PI(3)Kinase known as VPS34, PtdIns(3)P can also be found in the intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) (Gillooly, Morrow et al. 2000). PtdIns(3)P serve as membrane organizers for EEs, recruiting , in cooperation with other proteins, effector proteins for fusion such as EEA1, Rabenosyn-5 and rabankyrin-5 (Gaullier, Ronning et al. 2000; Nielsen, Christoforidis et al. 2000; Lawe, Chawla et al. 2002; Schnatwinkel, Christoforidis et al. 2004). These effectors along with others, such as EEA1 and Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), are able to bind to PtdIns(3)P through their FYVE- and PX-domains (Stenmark, Aasland et al. 1996; Gillooly, Morrow et al. 2000; Lawe, Patki et al. 2000). These proteins, recruited by the help of PtdIns(3)P, are proteins involved in several functions, some of these being cargo sorting by the assistance of Hrs (Raiborg, Bache et al. 2002; Sachse, Urbe et al. 2002), involved in sequestration of cargo into CCPs, that assist in the recruitment of components of the endosomal sorting complex required for transport (ESCRT) machinery (Katzmann, Stefan et al. 2003). SARA, a protein involved in TGF-beta/Smad signaling (Itoh, Divecha et al. 2002), and SNX1 and SNX2, sorting nexins that are involved in degradation of epidermal growth factor receptor (EGFR) and retrograde transport from LEs to TGN. PtdIns(3)P also assist in the recruitment of the motor protein KIF16-B which connects EEs to kinesin (Hoepfner, Severin et al. 2005).

Ptd(3,5)P_2, found on LEs, moreover participate in several organelle specific processes. Ptd(3,5)P_2 is mainly synthesized from the EE precursor PtdIns(3)P by the action of the kinase PIKfyve (Gary, Wurmser et al. 1998; Jefferies, Cooke et al. 2008). PtdIns(3)P are specifically localized to the EE membrane, this secures that the newly synthesized Ptd(3,5)P_2 is aggregated at the LE membrane, as the conversion of PtdIns(3)P to Ptd(3,5)P_2 is closely linked to the maturation of EE to LE. In yeast it has been reported to be involved in cargo-selective sorting to ILVs (Friant, Pecheur et al. 2003). The important task of retrograde recycling from LEs to the TGN is also helped by Ptd(3,5)P_2 as it is an important player in the effectors responsible for membrane fission.

The five other PtdIns(x)P_x are all involved in highly specific cellular processes, ranging from exocytic trafficking, the formation of CCPs, the ability to be altered into signaling lipids or the

ability to be cleaved to generate second messenger molecules (Lassing and Lindberg 1985; Ford, Pearse et al. 2001; Wang, Wang et al. 2003; Frederick, Mattiske et al. 2005).

1.2.3. Arf GTPases

Arf GTPases are similar to Rabs in many ways: Both are dependent on GEFs to switch to their active GTP-bound state, and both have GAPs to assist in their inactivation. They are also both assigned to their distinctive set of organelle membranes and exert their function in various roles ranging from coat protein recruitment, cargo sorting, activation of enzymes and cytoskeletal interactions. One of the major differences separating them, however, is the way in which they are recruited to membranes. Arf GTPases lipid anchor domain is dependent on a conformational change in its family specific interswitch domain (Pasqualato, Renault et al. 2002). In contrast to Rabs, which cover up their lipid anchor, the C-terminal prenyl group, by the aid of GDI (Shapiro and Pfeffer 1995), the Arf GTPases lipid anchor, and an N-terminal amphipathic helix, is tucked inside a groove in the protein itself (Goldberg 1998; Pasqualato, Renault et al. 2002).

1.2.4. Sorting Nexins

A group of membrane proteins containing a SNX-Phox homology (PX) domain have been named SNXs (Ponting 1996). The SNX-PX domain is able to interact and bind to phosphoinositides which are enriched in specific membranes; including to the SNX-PX domain they also contain domains that are able to interact with other proteins and lipids (Kanai, Liu et al. 2001). Sorting towards degradation, internalization and endosomal recycling are some of the functions that have been suggested. SNX1, the first mammalian SNX that was identified, was first shown through overexpression to down-regulate the amount of active EGFR found on the cell surface (Kurten, Cadena et al. 1996), but later experiments ruled this out as an artifact. SNX1 has instead been connected to the retrograde transport in cooperation with SNX2 as they form a complex that associates VPS26, VPS29 and VPS35, which makes up the retromer complex that is responsible for M6PR recycling back to the TGN (Griffin, Trejo et al. 2005; Rojas, Kametaka et al. 2007). Other SNXs perform functions in EE to RE transport (SNX3); regulation of endocytosis (SNX9; SNX17), trafficking (SNX15), and

regulation of degradation (SNX13) (Barr, Phillips et al. 2000; Xu, Hortsman et al. 2001; Zheng, Ma et al. 2001; Lundmark and Carlsson 2002; Stockinger, Sailler et al. 2002).

1.2.5. SNAREs

Membrane fusion has been shown to be highly dependent on the tethering proteins soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Membrane fusion instigated by SNAREs on separate membranes is thought to form a steady four helix bundle bringing the membranes closer and releasing free energy that can be used to drive the merging of the membranes (Figure 1-5).

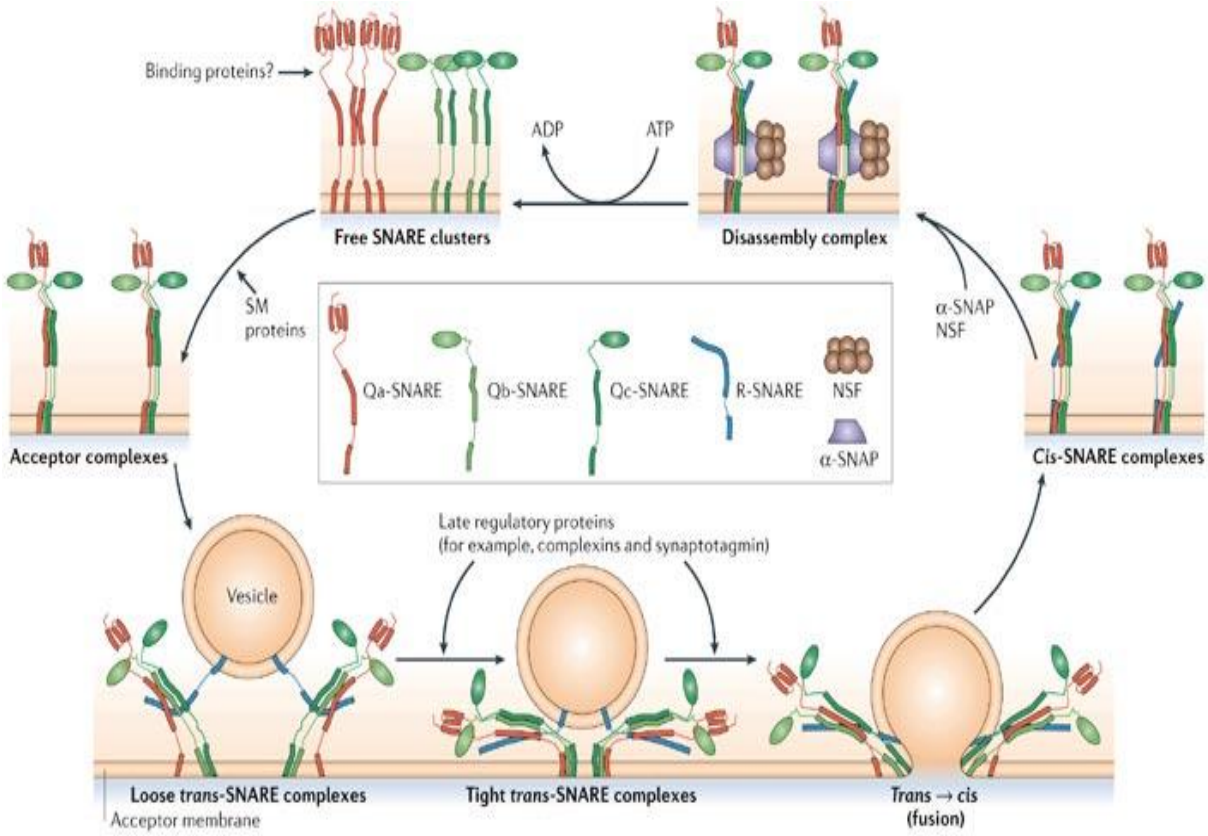

FIGURE 1-1.2-3: **THE SNARE MACHINERY.** STARTING IN THE UPPER LEFT CORNER ONE CAN SEE THE INDIVIDUAL SNARES BEFORE THEY ASSOCIATE INTO THE ACCEPTOR COMPLEX SEEN ON THE FAR LEFT. IT IS THOUGHT THAT THIS ASSEMBLY MIGHT BE DUE TO THE PRESENCE OF BINDING PROTEINS. THE ACCEPTOR COMPLES ARE ABLE TO INTERACT WITH THE R-SNARE ON AN INCOMMING VESICLE AND BRING THIS VESICLE IN CLOSE CONTANT WITH THE ACCEPTOR MEMBRANE. THIS FORCED PROXIMITY IS SUGGESTED TO OPEN A PARTLY FUSED PORE BETWEEN THE MEMBRANES, RESULTING IN THE INCORPORATION OF THE VESICLE MEMBRANE INTO THE ACCEPTOR MEMBRANE. AFTER THE SNARES HAVE PERFORMED THEIR FUNCTION THEY NEED TO BE RETURNED TO THEIR ORIGINAL STATE. N-ETHYLMALEIMIDE-SENSITIVE FACTORS AND (SOLUBLE N-ETHYLMALEIMIDE-SENSITIVE FACTOR ATTACHMENT PROTEIN) ARE RESPONSIBLE FOR THIS AND THE SNARES ARE NOW ABLE TO PERFORM THEIR FUNCTION ONCE AGAIN. (Jahn and Scheller 2006).

SNAREs are divided into Q- and R-SNAREs (Reclassified from T(arget)-SNAREs and V(esicle)-SNAREs. These terms are used, but are not as specific). Q-snares are usually found on the membrane, which a target vesicle is destined for, while the R-SNAREs are found on the transport vesicles (Fasshauer, Sutton et al. 1998). Individually they serve no function, but through SNARE motifs the individual Q-SNARES are able to associate with each other forming an acceptor-complex (Fasshauer, Otto et al. 1997). This acceptor complex can go on to affiliate itself with the SNARE motif of an R-SNARE leading to a highly stable four-helical core-complex (Fasshauer, Bruns et al. 1997; Rice, Brennwald et al. 1997). The acceptor complex and the R-SNARE motif interact loosely through the N-terminal region of the SNARE motifs (Chen, Tomchick et al. 2002). This association forms a loose *trans*-SNARE complex, but the interactions of regulatory proteins like complexins and synaptotagmin "zip up" the loose connection, making a stable tight *trans*/*cis*-SNARE complex and opening a hemifused pore in the membrane where the outer layer of both membranes associate with each other (McNew, Weber et al. 1999; Pabst, Margittai et al. 2002; Xu, Zhang et al. 2005). The stable *cis*-SNARE complex is recycled from the membrane by the catalytic action of an AAA+ N-ethylmaleimide-sensitive factors (NSF) protein, aided by its cofactors known as SNAPs (Zeng, Ren et al. 1999).

Since SNAREs are responsible for the fusion of transport vesicles to different target membrane enclosed organelles; they have to be sorted so that each organelle has their functional repertoire of SNAREs located on their membrane. The PM is for instance associated with the SNARES Syntaxin-1, Syntaxin-2, Syntaxin-4, SNAP-23 and SNAP-25. The Golgi membrane on the other hand, has Syntaxin-5 and VAMP4 imbedded in its membrane. Not all SNAREs are this rigid in their organelle localization, and even if they are organelle specific they can be found in intermediate membrane compartments in their recycling process. Even though different membranes may have their distinct repertoire, it is unclear how much they contribute to the fusion specificity (Hong 2005).

1.3. The Endo-Lysosomal Pathway.

1.3.1 Early Endosomes

Vesicles originating from the PM through endocytosis are likely to converge and fuse at the point EEs. EEs are the main sorting organelle in the endocytic pathway, responsible for determining the destination of most cargoes internalized. EEs can be recognized as pleomorphic organelles with a distinct morphology consisting of tubules at an early stage. The intraluminal environment has a slightly acidic pH (Maxfield and Yamashiro 1987) and a distinct set of membrane proteins that ensure organelle identity while having specific roles in sorting, trafficking and maturation. EEs are prone to undergo homotypic fusion with incoming endocytic vesicles and other EEs, increasing the concentration of internalized content (Gruenberg, Griffiths et al. 1989). The morphology is complex and there are variations as to where in the endosomal pathway the EEs are, from early EEs associated with thin tubular sections that average 0.06 µm in diameter (Gruenberg, Griffiths et al. 1989), to becoming spherical vesicles approximately 0.4 µm in diameter (Gruenberg, Griffiths et al. 1989) that can grow to become 0.8-1.0 µm preceding maturation (Rink, Ghigo et al. 2005; Driskell, Mironov et al. 2007).

The morphology is relevant to the task at hand; the tubules are exposed with different distinctive domains for recycling routes, making highly specific recycling possible. A recognizable selection of Rab GTPases is for instance detected on various domains on the EEs according to their function. Rab4 positive tubular domains on the early endosome are for example connected to direct recycling of cargo back to the PM (Van Der Sluijs, Hull et al. 1991; Daro, van der Sluijs et al. 1996; Mohrmann and van der Sluijs 1999). In addition to Rab4, Rab22 is also present on the EEs membrane. Rab22's role is reported to be an effector responsible for recruiting Rabex-5 to the membrane of EEs, which is an effector for the recruitment of Rab5 (Zhu, Liang et al. 2009). Rab5 is the most evident of the Rab GTPases found on EEs and its functions are many and only a few have been mentioned earlier. First activated by the GEF rabex-5 to the membrane of EEs, GTP-bound Rab5 itself is able to recruit Rabaptin-5 (Stenmark, Vitale et al. 1995), which in turn promotes further recruitment of Rabex-5, inducing a positive feedback loop for Rab5 (Lippe, Miaczynska et al. 2001). This is important for creating a stable amount of GTP-bound Rab5, and ensures the identity of EEs

and also later on as Rab5 is an important factor in the conversion of EEs to LEs. There have also been some reports of Rab15 domains found on EEs as well as on recycling endosomes (RE) and a suggested role in the recycling of cargo via this route, but these are not extensively researched and the role of Rab15 is likely to be clearer in the future. (Zuk and Elferink 1999; Zuk and Elferink 2000).

Another effector protein on EEs membrane is Rabenosyn-5. Rabenosyn-5 binds to the membrane in much the same way as EEA1, via a FYVE-domain and also active Rab GTPases, in this case both Rab5 and Rab4 (de Renzis, Sonnichsen et al. 2002). Rabenosyn-5 likely serves its role in the recycling from EEs in both the direct route and the RE mediated route (de Renzis, Sonnichsen et al. 2002; Naslavsky, Boehm et al. 2004).

As previously mentioned VPS34 or the PtdIns(3)P-kinase is also a noteworthy protein associated with EEs and is responsible for the membrane specific production of PtdIns(3)P (Christoforidis, Miaczynska et al. 1999). As mentioned earlier, PtdIns(3)P, in combination with GTP bound Rab5, is responsible for the recruitment of EEA1 to the EE membrane through EEA1's FYVE-domain (Lawe, Chawla et al. 2002). EEA1 is an important effector in the docking/fusion machinery (Simonsen, Lippe et al. 1998) that coordinates its function with SNAREs Syntaxin 6 and Syntaxin 13 (Mills, Urbe et al. 2001).

During the last decade there have been several studies indicating that endocytic organelles play a role in signaling. Some of the first indications were the observations that EEs acted as carriers of activated tyrosine kinase A receptors that interacted with signaling molecules of different pathways (Howe, Valletta et al. 2001; Delcroix, Valletta et al. 2003). The signaling proteins APPL1 and APPL2 are found on a subpopulation of EEs. These proteins are important for signaling in cell proliferation and require Rab5 to function properly (Miaczynska, Christoforidis et al. 2004). APPL1 and APPL2 react to epidermal growth factor and oxidative stress. As a response they will exit the EEs and relocate to the nucleus where they promote chromatin remodeling and gene expression, actively stimulating efficient cell proliferation (Miaczynska, Christoforidis et al. 2004). The other compartments of the endocytic pathway have also been implied in various signaling pathways (Lefkowitz and Shenoy 2005; Hisata, Sakisaka et al. 2007; Moore, Milano et al. 2007; Sadowski, Pilecka et al. 2009).

The formation of ILVs begins at an early endosomal stage, however, an increased rate of formation and an ILV filled lumen is associated with LEs. The biogenesis of these is therefore described in a more detailed manner in the section below. ILVs are produced by domains on the EEs that are rich in Clathrin and the proteins of the ESCRT machinery (Raiborg, Bache et al. 2002; Sachse, Urbe et al. 2002). The ESCRT machinery is responsible for the ILV formation and the internalization of ubiquitinated cargo into these. Other notable features about the EEs are their mildly acidic pH that starts around 6.8 but is reduced to around 5.9 in an EE about to mature (Maxfield and Yamashiro 1987).

1.3.2 Late Endosomes

Due to the increased number of ILVs in LEs, they are also commonly named multivesicular bodies (MVBs). The large number of ILVs is a distinct marker for LEs morphology (Cooney, Hurlburt et al. 2002), however this is just one of many morphological characteristics of LEs. LEs contain specific coat proteins, such as the small GTPase Rab7 and a distinct set of lipids ranging from triglycerides, cholesterol esters and unique phospholipids such as PtdIns(3,5)P and lysobiphosphatidic acid (LBPA). They have a pH ranging from 6.0 to 4.9 (Maxfield and Yamashiro 1987) and are localized to the perinuclear region of the cell. LEs are capable of undergoing homotypic fusion, and are also able to heterotypically fuse with lysosomes generating hybrid organelles (Luzio, Rous et al. 2000). In hybrid organelles the ILVs come in contact with the lysosomal degradative machinery. The hybrid organelles have certain endosomal markers such as M6PR (Griffiths 1989).However these are lost when the hybrid organelles undergo a maturation process, becoming a stable lysosome devoid of late endosomal markers and especially M6PR (Pryor, Mullock et al. 2000).

The advantages for the cell to cluster certain macromolecules into ILVs are numerous. Signaling receptors embedded in the membrane lose their signaling capabilities once they are removed from the cytosolic environment. Cargo internalized into ILVs is more accessible for hydrolase activity once the LE fuses with lysosomes. Additionally, proteins that exist in ILVs are suggested to have a role in lipid hydrolysis. However, different cells have evolved additional roles for ILVs other than the compartmentalization of macromolecules destined for degradation. Using the ILVs in exocytic events are for instance one mechanism found in synaptic cells and immune cells (Simons and Raposo 2009), like mature dendritic cells, which

secrete MCH II in exosomes to create an extracellular immune response (Buschow, Nolte-'t Hoen et al. 2009). Microautophagy is another process in which cytosolic components are taken up in ILVs in either a selective or unselective manner (Sahu, Kaushik et al. 2011). The process of “backfusion” is a suggested event where ILVs fuse with the limiting membrane of LE. It is suggested that internalized membrane proteins may use this to be recycled back to the limiting membrane so that they can be further recycled to TGN or other compartments (Kleijmeer, Ramm et al. 2001).

ILVs are formed by the involvement of a repertoire of different effector proteins, the most important being the members of the ESCRT complexes, these including the sub units ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. Together they form the machinery needed for the cell to sort, concentrate and internalize cargo into ILVs. Hrs is an important factor for the assembly of the ESCRT complex. Hrs binds to PtdIns(3)P on EEs (Raiborg, Bremnes et al. 2001) and is responsible for recruiting Clathrin to the membrane (Raiborg, Bache et al. 2001). Additionally it interacts with the STAM subunit, which also interacts with Clathrin, and together they form the ESCRT-0 complex (McCullough, Row et al. 2006). Both Hrs and STAM have ubiquitin-binding domains which interact with macromolecules that have been polyubiquitinated, a specific tag on macromolecules destined for degradation in lysosomes (Hofmann and Falquet 2001; Raiborg, Bache et al. 2002; Mizuno, Kawahata et al. 2003; Mayers, Fyfe et al. 2011). Consequently of the assembly of ESCRT-0 is the membrane recruitment of ESCRT-I, ESCRT-II and ESCRT-III by protein interactions between the complexes (Katzmann, Stefan et al. 2003; Teo, Perisic et al. 2004; Teo, Gill et al. 2006). ESCRT-I and ESCRT-II will further concentrate the ubiquitinated cargo into newly formed membrane invaginations through their ubiquitin-binding domains (Babst, Katzmann et al. 2002; Bilodeau, Winistorfer et al. 2003). ESCRT-III will then catalyze the scission of the inward oriented bud and create a fully formed ILV (Wollert, Wunder et al. 2009). The ESCRT complexes are then recycled back into cytosol by the action of AAA ATPase VPS4 that releases the different subunits from the limiting membrane of the EE/LE (Babst, Wendland et al. 1998; Lata, Schoehn et al. 2008).

The small GTPase Rab7 is one of the most distinct membrane proteins of LE. Some of its functions being e.g. regulation of motility, where Rab7 is able to promote minus-end directed transport of the LE by interacting with RILP and (oxysterol-binding protein) related

protein (ORP1L), mediating a connection to the dynein/dynactin motor protein complex (Johansson, Rocha et al. 2007). Rab7 is capable of promoting plus-end directed transport of autophagosomes, likely through the rab7- interacting protein FYVE and coiled coil domain containing 1 (FYCO1) which in turn is thought to recruit a kinesin motor (Pankiv, Alemu et al. 2010). Rab7 is also involved in the recruitment of the retromer to the LE membrane (Rojas, van Vlijmen et al. 2008). The retromer is essential for LE to TGN transport of the LE membrane associated M6PR and interacts with Rab7 through its VPS26 domain (Seaman 2004; Rojas, van Vlijmen et al. 2008). Rabring7 is a newly characterized effector of Rab7 found on LE. It has been reported to play a role in the trafficking of EGFR to lysosomes for degradation (Sakane, Hatakeyama et al. 2007). Rab7 may also exert a role on the EE associated PtdIns(3)Kinase which has an important role in maintaining the stability of the EE membrane coat proteins (Stein, Feng et al. 2003; Stein, Cao et al. 2005). Rab7 additionally has an important role in the endosomal maturation process as it is required for the Mon1-ccz1 complex activity(Poteryaev, Datta et al. 2010).

1.3.3 Lysosomes

The last stop of the endo-lysosomal pathway is the lysosome. With a pH that can drop to levels as low as 4.5 (Yamashiro and Maxfield 1987), providing an acidic environment optimizing the activity of acid hydrolases that break down macromolecules to their basic components. According to the definition it is not recognized as a lysosome before all M6PRs have dislocated from the limiting membrane (Bright, Reaves et al. 1997) . The major input source for content destined for degradation by lysosomal activities occur by the fusing of LEs/MVBs to an existing lysosome creating a hybrid organelle, which encompasses characteristics from both compartments (Luzio, Rous et al. 2000). This hybrid organelle matures back into a lysosome in a manner that membrane proteins are recycled and retrieved until a distinguishable morphology is achieved (Luzio, Rous et al. 2000). Apart from fusing with LEs, lysosomes have also shown that they are able to fuse with autophagosomes, phagosomes and the PM.

Like EEs and LEs lysosomes depend on a distinct set of membrane and luminal proteins to fulfill its role in the endosomal pathway. Lysosome-associated membrane protein 1 and 2 (LAMP-1 and LAMP-2), lysosome integral membrane protein 2 (LIMP 2) and tetraspanin

CD63 are the membrane proteins assorted to lysosomes or the hybrid organelle. LAMP-1 and LAMP-2 are found mostly on the limiting membrane, and have been suggested to play a role in maintaining the lysosomal integrity and regulating lysosomal cell death (Kannan, Stewart et al. 1996; Fehrenbacher, Bastholm et al. 2008). LIMP2 is connected to the transport of soluble lysosome hydrolases in an M6PR independent pathway (Reczek, Schwake et al. 2007). CD63 is also found in the lysosomal lumen along with other tetraspanins. Tetraspanins are connected to the formation of MHC Class II positive ILVs, which are meant for secretion during immune responses (Trajkovic, Hsu et al. 2008; Pols and Klumperman 2009). The proteins mainly associated with lysosome's degradative capabilities, the acid hydrolases, are usually delivered to EEs and LEs from the TGN by association of the M6PR (Doray, Ghosh et al. 2002; Peden, Oorschot et al. 2004). The milder acidic environment of these compartments are enough to disassociate the acid hydrolase from the M6PR, however it remains inactive until it reaches the lysosomal compartment. Lysosomal membrane proteins can either get to the lysosome by an indirect route that goes via the PM or it can take the direct path to the LE compartment from TGN by associating with proteins such as AP3, ubiquitin ligase and GGA3 (Bonifacino 2004; Janvier and Bonifacino 2005).

The hybrid organelle needs to return to a lysosome specific state, where all markers and effectors from LEs that are to be recycled are removed. Failing to do so would result in the degradation of many receptor proteins, which should be recycled, and an ineffective luminal environment for degradation caused by an increased pH value and a shift of ion composition. Membrane embedded ATPase proton pumps and Ca^{2+}-channels work to bring the luminal environment back to the lysosome core state (Pryor, Mullock et al. 2000). And complexes such as the retromer are involved in the recycling of the M6PR back to TGN (Seaman 2004). It is only when all of M6PR have been removed from the limiting membrane that one distinguishes between a hybrid organelle and a lysosome (Bright, Reaves et al. 1997).

1.4. Early To Late Endosomal Maturation

The switch from early to late endosome is one of the most important events along the endocytic pathway. During the maturation the early endosomal function of recycling is reduced and the late endosomal compartment is altered in a way to efficiently deliver its cargo for degradation in the lysosomal compartment. These modifications include a new set of characteristic membrane associated proteins like Rab7, PtdIns(3,5)P_2 and specific snares, an increased formation of ILVs. A decrease of pH from values above 6 to 6.0 - 4.9 is observed, increased size and loss of tubular extension associated with the EE, motility associated functions and an altered concentrations of the ionic composition.

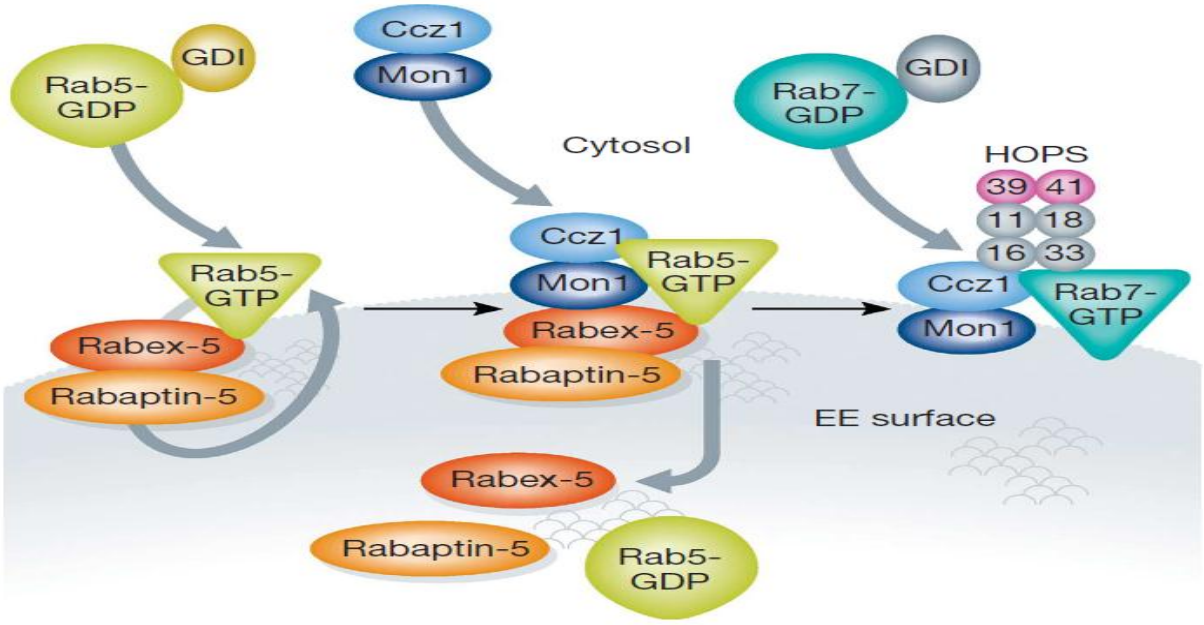

FIGURE 1.4-1: **SOME OF THE INVOLVED EFFECTORS IN THE RAB5/RAB7 CONVERSION**. RAB5 IS ABLE TO RECRUIT ITSELF ON TO THE MEMBRANE BY ASSOCIATING WITH EFFECTOR PROTEINS SUPPORTING ITS OWN FEEDBACK LOOP. HOWEVER, THIS FEEDBACK LOOP IS INACTIVATED IN AN UNKNOWN MANNER AS MATURATION BEGINS. SEVERAL PROTEINS HAVE BEEN IMPLICATED IN THE PROCESS OF MATURATION, BUT THE EXCTENT OF THEIR INTERACTIONS ARE FAR FROM FULLY UNDERSTOOD. THE COMPLEX CONSISTING OF MON1 AND CCZ1 HAVE JUST RECENTLY BEEN IDENTIFIED AS A CRUCIAL SWITCH IN MATURATION. IT WAS ALSO IDENTIFIED AS THE GEF FOR RAB7 IN MAMMALS, FURTHER INCREASING ITS IMPORTANCE IN MATURATION. (HUOTARI AND HELENIUS 2011).

Though it might be possible for endocytosed cargo to bypass EEs entirely (Payne, Jones et al. 2007) or be delivered to LEs by budding vesicles from EEs (Vonderheit and Helenius 2005), most cargo usually originates from endocytosis. If they are not recycled back to the PM or RE, they have to wait for the next step where EE matures into LEs (Rink, Ghigo et al. 2005).

This maturing process is a highly complex event, most easily defined by the loss of Rab5 GTPase and the subsequent attachment of Rab7 GTPase (Rink, Ghigo et al. 2005). This is influenced by a series of different effector proteins shown in Figure 1-6, one of the most important being SAND-1/Mon1, which was identified as the switch in the early to late transition (Poteryaev, Datta et al. 2010). It has been implied that in a sequence of reactions where Mon1 binds to activated Rab5 (Kinchen and Ravichandran 2010) then another effector protein binds to this complex. Ccz1 is recruited to Mon1, forming a complex that in turn recruits Rab7 to the membrane. This Mon1-ccz1 complex acts as the GDF displacing the GDI from cytosolic inactive GDP-bound Rab7 (Kinchen and Ravichandran 2010). The proper protein interaction responsible for GEF activity for Rab7 has yet to be revealed, but newer research indicates that the Mon1-Ccz1 complex, and not Vps39, is responsible for nucleotide exchange in some way (Nordmann, Cabrera et al. 2010) VPS39 was previously suggested for this function on a basis of observations made in yeast(Wurmser, Sato et al. 2000), but failed to show any kind of interaction in mammalian cells (Nordmann, Cabrera et al. 2010).

The phosphatidylinositol-3-phophate, a characteristic lipid only found on early endosomes (Gillooly, Morrow et al. 2000), has been implemented in recruiting Mon1 to EEs so that it can bind to Rab5 (Poteryaev, Datta et al. 2010). This was indicated by treating cells with the PI(3)P kinase inhibitor Wortmannin, resulting in the abolished recruitment of Mon1 to EEs (Poteryaev, Datta et al. 2010). This led to speculations that the concentrations of these lipids may be essential for determining when maturation occurs. The Mon1-Ccz1 complex is also thought to interact with the homotypic fusion and vacuole protein sorting (HOPS) complex (Nordmann, Cabrera et al. 2010) and via this interaction cause disassociation of Rabex-5 from the EE-membrane. This is thought to disrupt the positive feedback loop, thus ending the recruitment of Rab5 to EEs, allowing Rab7 to populate the membrane. Rab5 needs to be inactivated by specific GAPs, and to date both RabGAP-5 and Rab GTPase activating protein TBC-2 have been identified to serve this role (Haas, Fuchs et al. 2005; Chotard, Mishra et al. 2010). RabGAP-5 is reported to bring about a loss of EEA1 from EEs (Haas, Fuchs et al. 2005), while TBC-2 is thought to be dependent of Rab7 for membrane localization and function (Chotard, Mishra et al. 2010).

Another notable event connected to the endosomal maturation is the conversion of the vacuole and a novel endosomal tethering (CORVET) complex to HOPS. The VPS-C core

complex is built up by four members of the VPS family, these being the VPS11, VPS16, VPS18 and VPS33 (Sato, Rehling et al. 2000; Seals, Eitzen et al. 2000). Together these VPS-C complexes are involved in cellular mechanisms involving degradation of endocytosed macromolecules, autophagy, cholesterol and lipid metabolism, antigen presenting and membrane fusion events (Seals, Eitzen et al. 2000; Wurmser, Sato et al. 2000; Levine and Klionsky 2004; Wilkin, Tongngok et al. 2008). CORVET associates with the small GTPase Rab5 and thus EEs and is built up by VPS3 and VPS8 in addition to the VPS-C core complex (Peplowska, Markgraf et al. 2007). Its specific roles are yet to be properly understood, but it is likely to be involved in tethering (Peplowska, Markgraf et al. 2007), and retrograde transport (Bryant, Piper et al. 1998). The HOPS complex is associated with the VPS39 and VPS41 in addition to the VPS-C core complex (Nakamura, Hirata et al. 1997). VPS39 has been reported to be the GEF for Rab7 in yeast, however, it does not assert this function in mammals, and the GEF activity has been shown to reside in the Mon1-Ccz1 complex, so the function for VPS39 is still undecided. VPS41 on the other hand has been shown to be an effector for Rab7-specific activities and is likely to have a role in AP-3 vesicle biogenesis (Darsow, Katzmann et al. 2001). The underlying function and mechanism behind the "maturation" of the CORVET complex into the HOPS complex is still largely unknown.

Parallel to the Rab5/Rab7 conversion is the exchange from PtdIns(3)P to PtdIns(3,5)P_2. PtdIns(3)P is continually recruited to the limiting membrane of the EEs by the action of VPS34 (Schu, Takegawa et al. 1993), a kinase recruited with the help of Rab5 and p150 (Murray, Panaretou et al. 2002). VPS34, with its regulators p150 and beclin1 (Funderburk, Wang et al. 2010), associate with the HOPS complex activating protein UVRAG (UV Radiation resistance associated gene) (Itakura, Kishi et al. 2008). UVRAG is an enhancer of PI(3)K activity and is linked to the maturation process via its interaction with the HOPS complex (Liang, Lee et al. 2008). Related to this is the protein Rubicon (Sun, Westphal et al. 2010), acting as a negative regulator towards UVRAG. Binding of GTP-bound Rab7 to Rubicon however, sequesters Rubicon from UVRAG, allowing UVRAG to associate with the HOPS-complex. The PtdIns(3)P on the membrane of EEs are directly involved in its conversion during maturation by the consequence that they, through their FYVE-domain binding capabilities, recruit the phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), which is

responsible for the conversion of PtdIns(3)P to PtdIns(3,5)P_2 to the membrane (Gary, Wurmser et al. 1998). PIKfyve works in a complex with its activator ArPIKfyve and Sac3 known as the PAS-complex, providing optimal PIKfyve functionality (Sbrissa, Ikonomov et al. 2008). Another role for PIKfyve has also been suggested in processes such as retrograde transport from LE to TGN, where it is thought to interact with effectors from this transport route (de Lartigue, Polson et al. 2009; Ikonomov, Fligger et al. 2009). PIKfyve has also been connected to the regulation of ion channels to the PM by a site-specific phosphorylation of PIKfyve by a protein kinase, SGK1, affecting the activity of ion channels at the PM (Shojaiefard, Strutz-Seebohm et al. 2007; Strutz-Seebohm, Shojaiefard et al. 2007). Additional to this, PIKfyve has been thought to have a role involved in the process of acidification of LEs and lysosomes, but this role is largely unconfirmed. Lastly, it is worth mentioning that even though the ESCRT-0 complex initially uses PtdIns(3)P as an anchorage point for ILV formation, it is fully capable of binding to PtdIns(3,5)P_2 to maintain the compartmentalization of macromolecules into ILVs, a feature that makes the PtdIns conversion insignificant in this manner (Whitley, Reaves et al. 2003).

Rab7 then goes on to recruit its own effectors, like the LE-dynein linking protein RILP responsible for movement (Jordens, Fernandez-Borja et al. 2001; Johansson, Rocha et al. 2007; Progida, Malerod et al. 2007), retromer components responsible for retrograde transport from LEs to TGN (Arighi, Hartnell et al. 2004; Rojas, van Vlijmen et al. 2008), proteins required for tethering and fusion like the HOPS complex (Ostrowicz, Brocker et al. 2010) and more or less complete the conversion of the EE to a LE.

The maturation event is one of the most significant checkpoints in the endo-lysosomal pathway. The importance of this conversion, from EE to LE, is evident in terms of its complexity. The number of effectors and the amount of interactions related to this process is likely to increase as research towards this field is constantly increasing.

2. Aim Of Study

The endosomal system is the main traffic route for macromolecules and receptors for degradation. In this pleiomorphic system, interaction and progression through the pathway are maintained by the identity of the organelles. These compartments can be divided into the different organelles, EEs, LE, and lysosomes, by their specific localized membrane proteins. Progression from the EEs to LEs ensures directionality and destiny for the internalized molecules (Rink, Ghigo et al. 2005).

Fluorescently tagged Rab5, Rab7 and ctEEA1 were used to identify and measure the maturation as a loss of fluorescent coat. To better visualize the endosomal processes and interactions we will utilize the ability of known fusogenic proteins to increase the endosomal size. Ii and Rab5-wt and Rab5-Q79L will be expressed behind and inducible promoter in order to enlarge the endosomes (Bucci, Parton et al. 1992; Stenmark, Valencia et al. 1994; Stang and Bakke 1997).

The primary aim of this study was do develop an imaging method to detect any specific feedback mechanism from Rab7 to regulate the membrane dynamics of Rab 5. Furthermore we seek to develop a method to demonstrate an intra-endosomal feedback process and to identify the mechanism regulating coat detachment/attachment during endosomal maturation.

Specific Aims Of The Study:

A) Use fluorescently tagged Rab5-wt and Rab7-wt/Q67L/T22N to establish a method to effectively measure the maturation kinetics of Rab5 and to investigate the effect Rab7 has towards Rab5 during this process.
B) Make use of FRAP and fluorescently tagged Rab5-wt and Rab7-wt/Q67L/T22N to see what effect increased Rab7 expression has on the Rab5 coat prior to maturation.

3. Materials And Methods

3.1. DNA

The pcDNA3 expression vector, (Figure 3.1-1 A) with the Rab5-wt-mCherry inserts, was used for transient transfection.

The inducible pMEP4 expression vector (Figure 3.1-1 B) with the following inserts was used to control the expression of fusogenic proteins in order to create enlarged vesicles: ***Rab5-wt, Rab5-Q79L and Ii.***

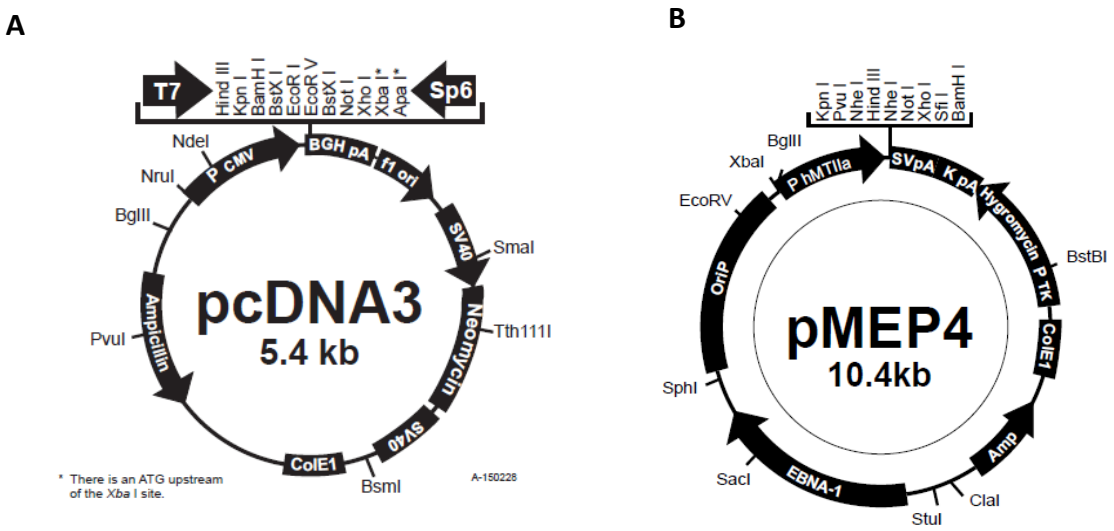

FIGURE 3.1-1: THE VECTORS USED FOR EXPRESSION OF INSERTED DNA. A) PCDNA3 WAS USED FOR THE TRANSIENT TRANSFECTION OF RAB5-WT-MCHERRRY. B) THE INDUCIBLE PMEP4 VECTOR WAS INSERTED RAB5-WT, RAB5-Q79L, AND II. THIS MADE IT POSSIBLE TO CONTROL THE FORMATION OF ENLARGED VESICLES. FIGURES AQUIRED FROM INVITROGEN.

The constructs with Rab7-wt-EGFP, Rab7-Q67L-EGFP and Rab7-T22N-EGFP was acquired from Cecilia Bucci's lab and has been previously described in (Bucci, Thomsen et al. 2000).

3.2. Cell culture

Madine-Darby Canine kidney strain II (MDCK) cell lines were used. Stable cell lines were created by stably transfecting the inducible vector into the cells. To ensure only cells carrying the construct prevailed, they were put on medium containing antibiotics. The cells were grown in complete medium: DMEM (Bio Whittaker) supplemented with 9% FCS (Integro),

2mM L-Glutamine, 25 U/ml Penicillin (Bio Whittaker) and 25 µg/ml streptomycin (Bio Whittaker). The cells were stored in a 6% CO_2 incubator at the stable temperature of 37^0C.

Hygromycin (Saveen & Werner) and G-418 (PAA) could be added in concentrations 0.3mg/ml and 1.0mg/ml respectively to the complete medium to create a selection medium towards cells not bearing the vectors (Shown in Figure 3.1-1) with the inserted resistance gene.

3.3. Preparations For Imaging

To prepare for imaging the cells were grown in chambered coverglass from Nalgene and Mattek, in a solution of complete medium at 37^0C. The cells were transiently transfected when they were 75-90% confluent using a Lipofectamine2000 (Invitrogen) Opti-Mem (Invitrogen) mix which was added to the cells. The medium was switched to DMEM with FCS and L-Glutamine (without antibiotics). Transfected cells were incubated with $CdCl_2$ 5-16 hours before imaging. Prior to the imaging the cells are washed with 1xPBS and medium is replaced with microscopy medium DMEM (with FCS, L-Glutamine, Penicillin and Streptomycin) without phenol red and 25mM HEPES, adjusted to pH 7,5.

3.4. Confocal Microscopy

To collect the data, two different microscopes were utilized.

The Andor Revolution XD spinning disc microscope and the Olympus Fluoview 1000 inverted. The Andor spinning disc platform used was the Olympus IX 71 with PlanApo N 60X/ 1.42 NA oil immersion objective. The spinning disc unit CSU22 was synchronized with an iXon^{Em+} 885 EMCCD camera. The 37^0C microscope chamber delivered by Solent scientific created a stable cellular environment. The spinning disc technology was provided by Nipkow, using a multiple pinhole disc. Exposure time was set to 500ms and samples were excited by an Argon/Krypton, Argon ion laser using the 488/568 lines.

On the Olympus Fluoview 1000 inverted microscope the used equipment were PlanApo x60/1.42 oil objective. The images were obtained by this objective, a SIM-scanner and a

photomultiplier tube. The samples were excited by a multi-line Argon laser (457, 488, 515nm) at 488nm for GFP; and a solide state laser (559nm) for mCherry.

3.5. Fluorescence Recovery After Photobleaching (FRAP)

FRAP is a microscope technique used to examine the kinetic properties of fluorescently tagged molecules. A designated area of a cell or organelle can be accurately targeted for bleached. The bleaching is achieved by focusing a high powered laser beam in a definite area for a short length of time, bleaching the fluorescently tagged molecules present in that area. The ensuing recovery of fluorescence can be interpreted to further comprehend the kinetics of a protein, lipid or other macromolecules.

In this assignment the Olympus Fluoview 1000 inverted microscope was used for FRAP. The setup was similar to what formerly mentioned. The bleaching of Rab5-wt-mCherry was achieved by splitting the 559 solid state laser 50/50 ADM at 100% laser intensity, where one half went to bleaching while the other was used for imaging. This made it possible to both bleach and capture an image simultaneously. Ii-induced enlarged vesicles positive for Rab5-wt-mCherry in cells co-transfected with the various Rab7 mutants were targeted for bleaching by a “tornado” scanner and bleaching time was 1000µS.

The FRAP data was normalized and corrected for bleaching (Pelkmans, Kartenbeck et al. 2001). Relative intensity of bleached area F_{FRAP} was calculated using the following equation.

$$F(t) = \frac{I_{total\ (0)}}{I_{Bleach(0)}} x \frac{I_{Bleach\ (t)}}{I_{total\ (t)}}$$

$I_{total\ (0)}$ is the total intensity of the cell prior to bleaching, $I_{Bleach(0)}$ is the total intensity of the bleached area before bleaching, $I_{Bleach\ (t)}$ is the intensity of the bleached area over time, and $I_{total\ (t)}$ is the intensity of the complete cell over time. T½ and immobile fractions were calculated by non-linear regression according to (Yguerabide, Schmidt et al. 1982; Lippincott-Schwartz 2001)

3.6. Video Editing And Quantitative Analysis

3.6.1. Analysis Software

For video and image analysis ImageJ software was used. ImageJ was used to measure the variation of intensity during maturation, co-localization and distribution throughout the experiments. The data collected through ImageJ was quantified and processed in Microsoft excel. Montages and edited videos were created using ImageJ.

3.6.2. Maturation Analysis

The maturation analysis was done by defining the maturing EE into a spherical region of interest (ROI) just outside the limiting membrane. The intensity of the declining Rab5 and ascending Rab7 were measured this way, frame by frame. This increased the accuracy of the results as doing it this way reduces the interference from incoming vesicles. The measured intensities were then transferred into excel and standardized towards 1 and 0. The results were synchronized around the data points closest to 0.5 measured in relative intensity. By synchronizing around this halfway point, we hoped to effectively reduce the standard deviation (StdDev) for both the M½ and M_{end}.

3.6.3. Co-localization Analysis

Co-localization was measured by defining EEs as ROIs in one of the channels (Rab5/red). The way this was done was first to: Subtract background to reduce unnecessary noise, convert the image into binary (black and white), threshold the image to remove as much signals, observed not likely to be EEs, and then lastly define the ROIs into a ROI-manager. These ROIs were then overlapped with the second channel (Rab7/green). By directly observing the intensity within these ROIs, physically observing what is thought to be a co-localizing LE, one could calculate the percentage of co-localization.

3.6.4. Distribution Analysis

Distribution was calculated by defining all intensities over a certain level as single points. First the image was converted into binary, and then ImageJ was used to find maxima and to display these as single points. By defining a narrow region close to the membrane and nucleus of the cell one could easily count the number EE as single points according to their position. Because of the inconsistency of the shape of the cells, these regions were manually sectioned. The nucleus region was first removed, and then the polygon selection tool was used to define the perinuclear region. The same procedure was done to define the region close to the PM and the number of single points was measured within these regions.

4. Results

4.1.1. Introduction

The maturation of EEs to LEs, as defined by the conversion of GTP-bound Rab5 to GTP-bound Rab7, is a tightly controlled process involving several effector proteins (Poteryaev, Datta et al. 2010). We believe however, that there is a mutual feedback loop between Rab5 and Rab7 during maturation and Rab7 may control the Rab5 coat dynamics on EEs. To further investigate the role of Rab7 in this process we have looked for any alterations in the binding dynamics of Rab5 during the maturation process in cells expressing different mutants of Rab7. More precisely we looked for a change in Rab5 uncoating kinetics.

In order to better measure the coat kinetics of Rab5 by the confocal microscopy we increased the size of the endosomes by three well-known methods. To achieve enlarged endosomes, an overexpression of Rab5; the positive dominant Rab5-Q79L; and the overexpression of Ii were used. It has been shown in previous experiments in Bakke lab that Ii-induced enlarged vesicles (ILEVs) are formed by specific fusogenic properties of Ii, and that these ILEVs can be used to study endocytic interactions (Landsverk, Barois et al. 2011). Enlarged endosomes serves as a great tool as the increased size makes it easier to observe and analyze the interactions during maturation. The motility of the compartment is also reduced as a result of the enlargement and this gives a greater accuracy, as the endosomes are less likely to move in and out of focus.

To investigate Rab5 binding dynamics during maturation and the complex interaction between Rab5 and Rab7 cells were co-transfected with Rab7-wt-EGFP; the dominant negative Rab7-T22N-GFP; and the dominant positive Rab7-Q67L-EGFP. The early endosomal membrane dynamics of Rab5 were measured by live cell imaging where the membrane dynamics were analyzed by two different approaches. These were done by live fluorescent intensity measurements, analyzing the coat detachment and FRAP experiments on the Rab5-wt-mCherry positive endosomes. To measure the maturation as function fluorescent loss of Rab5-wt-mCherry, three measurement points were defined. M_{start} represents the time point of imitated maturation, as the intensity of the Rab5-wt-mCherry coat starts detaching. M½ represents the time point where the maturation process is halfway through, and is

synchronized to the point of 0.5 measured in relative intensity. M_{end} represents the end of maturation, estimated to the point where the rapid decline of Rab5-wt-mCherry intensity halts.

The FRAP experiments were performed in cells with enlarged endosomes induced by the overexpression of Ii. Similar to previously described experiments the cells were transfected with Rab5-mCherry and co-transfected with the different Rab7s. This was done to investigate the effect Rab7 had on Rab5 prior to maturation and to establish a T½-recovery as a measurement for the Rab5-wt-mCherry on-off cycling. If one could detect any notable change in the recovery time of Rab5 on the EEs, then this may indicate a Rab7 control on the Rab5 coat dynamics before maturation.

4.1.2. Enlarged Endosomes, A General Description

The enlarged vesicles induced by the various fusogenic proteins show different morphological characteristics. The Rab5-wt-induced vesicles are about of 2.0 µm on average in diameter (Bucci, Parton et al. 1992), The Rab5-Q79L-induced vesicles average to about 3.5 µm in diameter (Stenmark, Parton et al. 1994), and the Ii-induced vesicles are about 2.0-5.0 µm in diameter (Stang and Bakke 1997). The Rab5-Q79L, that is defined to its active GTP-bound state, produces enlarged cytosolic compartments that carry distinct characteristics from distinct compartments in the cytosolic pathway (Wegner, Malerod et al. 2010). These altered characteristics could be endocytic compartments positive for the EE marker EEA1 and the LE marker Rab7 (Figure 4.1.2-1 B), as well as published results that the expression of Rab5-Q67L affects the on-off kinetics of EEA1 (Bergeland, Haugen et al. 2008; Wegner, Malerod et al. 2010). In Figure 4.1.2-1 this is illustrated by the observation of co-localization between the EE-marker EEA1-mRFP and the LE-marker Rab7-EGFP in Rab5-Q79L-induced cells (4.2-1 B). This overlap and the altered kinetics (Bergeland, Haugen et al. 2008), are apparent weaknesses in the Rab5-Q79L-induced system for the study of the kinetics of membrane proteins in the endocytic pathway.

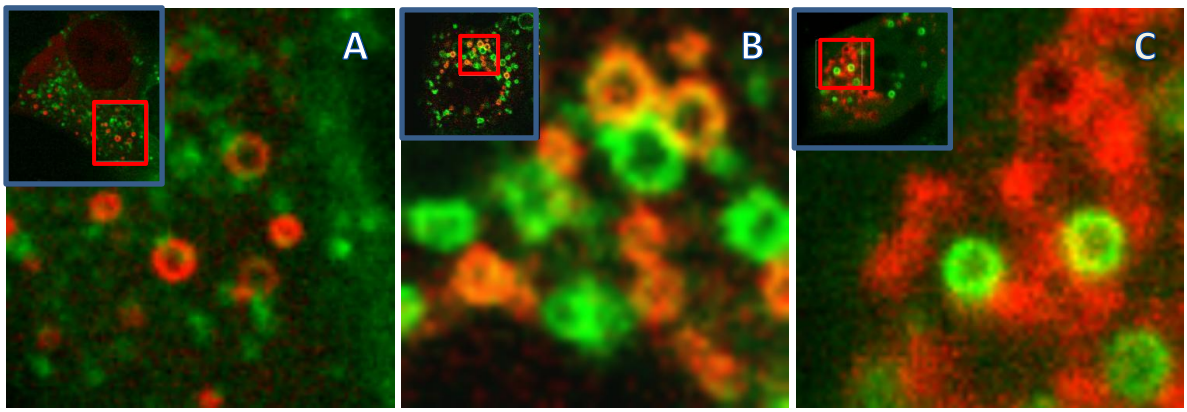

FIGURE 4.1.2-1: THE EE MARKER EEA1 AND LE MARKER RAB7 AND THEIR LOCATION DEPENDING ON THE VARIOUS SYSTEMS. A) EEA1-RFP & RAB7-WT-EGFP IN RAB5-WT-INDUCED CELLS, MIDDLE PICTURE B) EEA1-RFP & RAB7-WT-EGFP IN RAB5 Q79L INDUCED CELLS, AND C) EEA1-GFP & RAB7-WT-MCHERRY IN II-INDUCED CELLS.

The EEs lifetime is also affected by the system that is used. The lifetime for EEs induced by Rab5-wt is averaged to be 50 min (Landsverk, Barois et al. 2011), quite similar to the lifetime of Rab5-Q79L (data not shown). The lifetime of Ii-induced vesicles on the other hand, is about 2½ H (Landsverk, Barois et al. 2011).

4.2. Rab5-wt-mCherry And ctEEA1-GFP Dynamics During Maturation

EEA1 is a tethering agent recruited to the membrane of EEs by its interactions with Rab5 (Christoforidis, McBride et al. 1999). Since EEA1 is dependent on Rab5 for its localization to the EEs limiting membrane, its detachment is also closely linked to the loss of Rab5 during maturation as seen in Figure 4.2-1 and 4.2-2

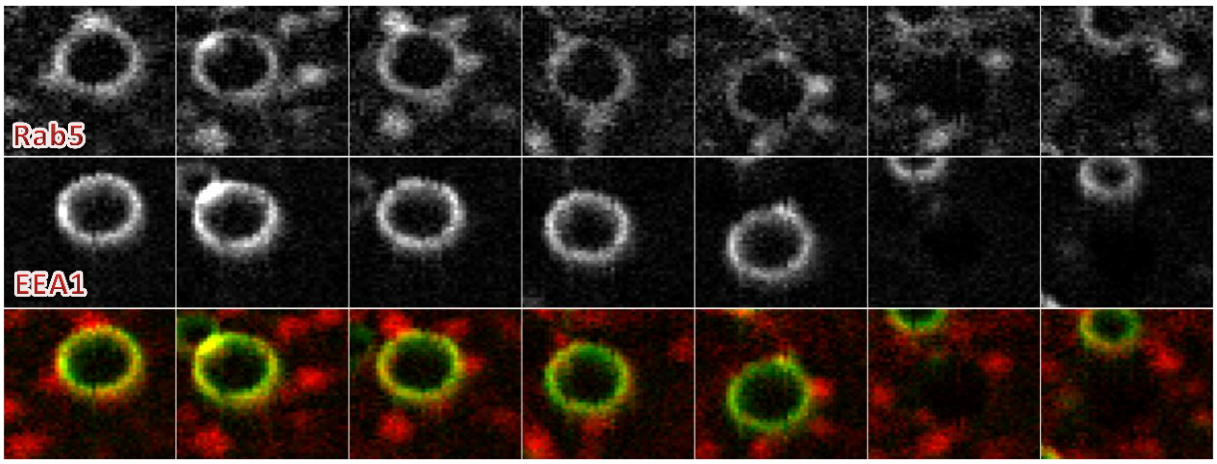

FIGURE 4.2-1: MATURATION OF A SINGLE ENDOSOME. THE INTENSITY OF RAB5-WT-MCHERRY IS HERE GRADUALLY REDUCED CONCOMITANTLY WITH EEA1-GFP

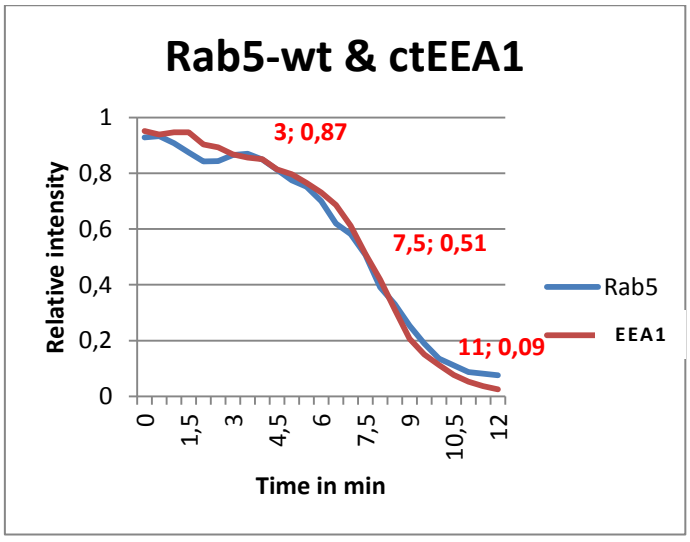

FIGURE 4.2-2: THE DECLINE OF RAB5 AND EEA1 IN A MATURING ENDOSOME. RAB5 AND EEA1 ARE BOTH LOST SIMULTANEOUSLY FROM THE LIMITING MEMBRANE OF AN II-INDUCED EE DURING MATURATION.

Ii-induced vesicles that are co-transfected with Rab5-wt-mcherry and ctEEA1-GFP provide evidence that the Ii-induced system do not disturb maturation dynamics (Movie 1). Both early endosomal markers are detached, and this is providing good grounds for the continuous use of the Ii-inducible system.

4.3. Rab5-wt-mCherry Dynamics During Maturation

Rab5 circulates between a GDP-bound cytosolic state and a GTP-bound membrane-bound state. Before one could proper investigate the possible effect of Rab7 exerted on membrane dynamics, one needed to characterize the Rab5-wt-mCherry dynamics in cells untransfected with Rab7. From this it would be clearer if an increased expression of Rab7-wt and the Rab7 mutants had any effect on the coat dynamics of Rab5 during maturation. The three individual systems were transfected with Rab5-wt-mCherry and induced to generate enlarged endocytic organelles and could be observed while they matured, visualized here in Figure 4.3.1. Imaging was then performed with a 30 sec interval between frames (Movie 2, Movie 3 and Movie 4) and the data was analyzed in ImageJ and these data are presented in Figure 4.3-2.

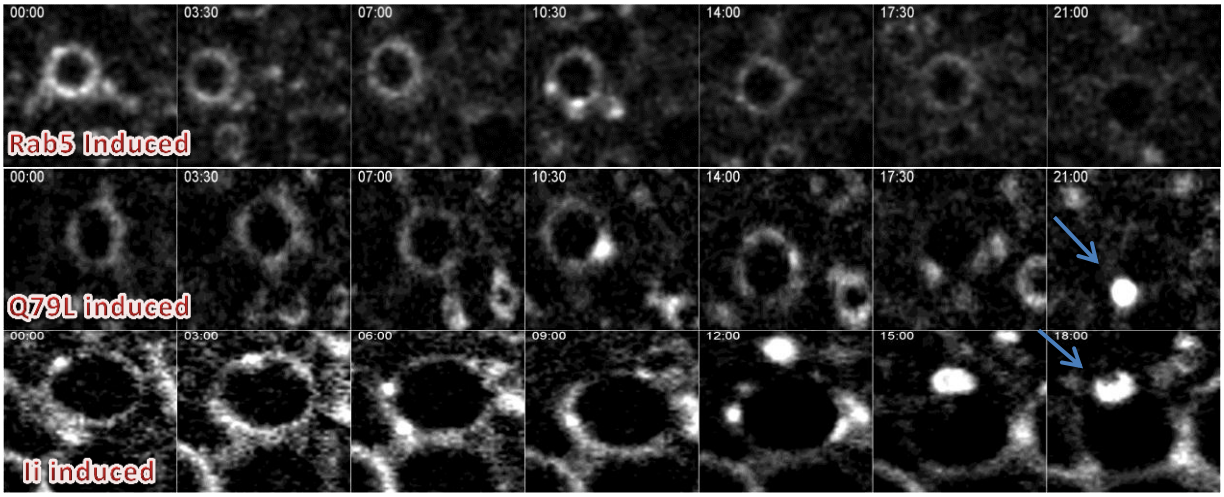

FIGURE 4.3-1: RAB5 MCHERRY MATURATION ON A SINGLE ENDOSOME. IMAGE SERIES SHOWING THE DECLINE OF RAB5, THAT IS PRESENT ON EES LIMITING MEMBRANE, FOR EACH INDIVIDUAL SYSTEM. VESICULAR INTERACTIONS FROM RAB5 POSITIVE VESICLES ARE ALSO VISIBLE IN ALL SYSTEMS. THE NOTABLE PRESENCE OF A RAB5 POSITIVE VESICLE (ARROWS) ASSOCIATED WITH WHAT IS THOUGHT TO BE A FULLY MATURED LE IS A REPETITIVE OBSERVATION (HERE SEEN IN THE LAST FRAMES OF THE Q79L/II-INDUCED IMAGE SERIES.).

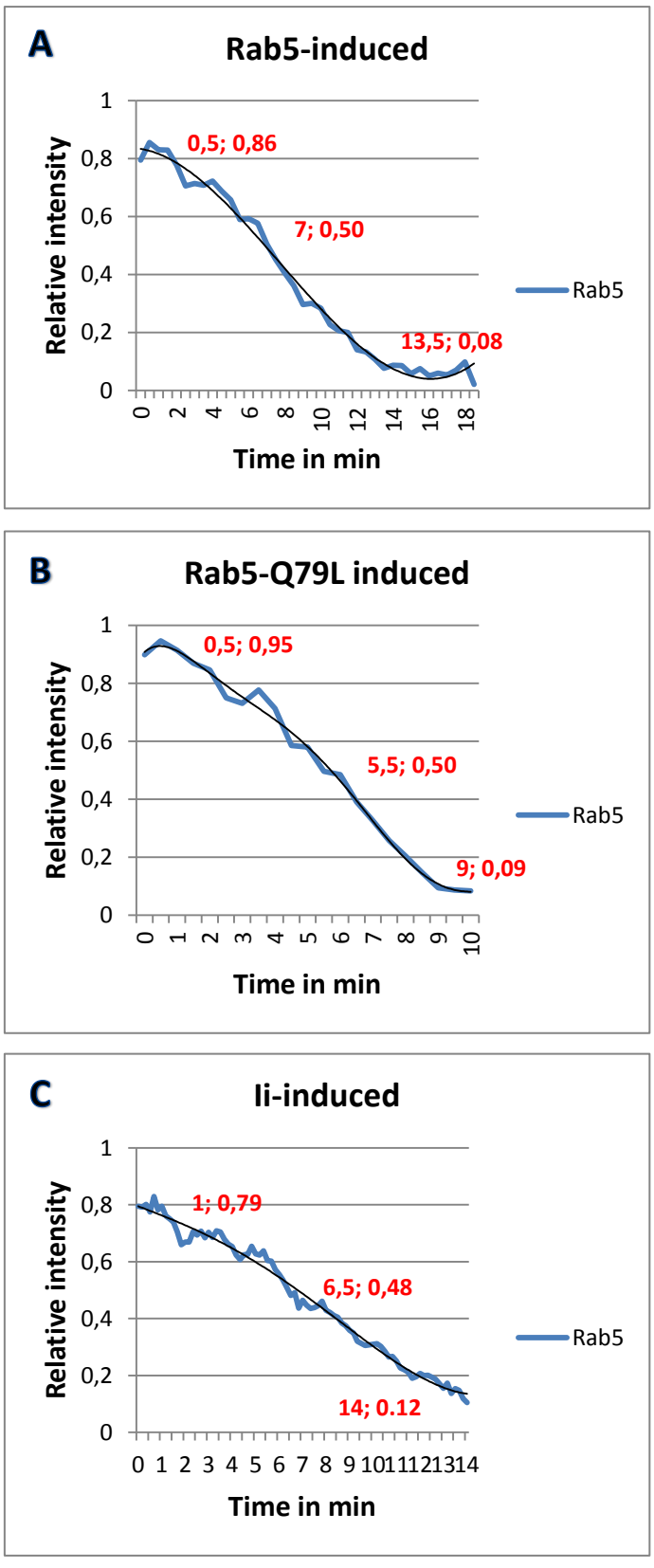

FIGURE 4.3-2: DATA SHOWING THE DECLINE OF RELATIVE INTENSITY OF RAB5 IN CELLS TRANSIENTLY TRANSFECTED WITH RAB5-WT-MCHERRY. ALL DATA ARE SYNCHRONIZED AROUND THE DATA POINT CLOSEST TO 0.5 RELATIVE INTENSITY (RI) A) RAB5-WT ENLARGED VESICLES (N=18), M_{START}=0.5 MIN/0.92 RI, M½= 7,0 MIN ± 1,6MIN/ 0.5 RI, AND M_{END}=13.5 MIN ± 1,1 MIN/ 0.09 RI . B) RAB5-Q79L ENLARGED VESICLES (N=9), M_{START}=0.5 MIN/0.95 RI, M½= 5.5 MIN ± 1.3 MIN/ 0.48 RI, AND M_{END}=9.0 MIN ± 1.0 MIN/ 0.09 RI. C) ILEVS (N=13), M_{START}=0.5 MIN/0.85 RI, M½= 6.0 MIN ± 1.2 MIN/ 0.48 RI, AND M_{FND}=14.0 MIN ±0.5/ 0.13 RI

As seen in Figure 4.3-2 and in Table 4.3.-1 below, the rate of Rab5 loss from maturing EEs differ depending on what system has been used to induce enlarged vesicles.

Table 4.3-1	**M½**	**M_{END}**
Rab5 induced	**6,5 min ± 1,6 min**	**13,0 min ± 1.1 min**
Rab5-Q79L-induced	**5,0 min ± 1.3 min**	**8,5 min ± 1.1 min**
Ii-induced	**5,5 min ± 1.2 min**	**13,0 min ± 0.5 min**

The M½ for the invariant chain and Rab5-Q79L-induced vesicles are quite similar. However, whereas the Rab5-Q79L vesicles maintain a steep drop throughout the maturation, the invariant chain induced vesicles' curve bends off and ends their maturation 4.5 min later. The Rab5 induced vesicles have a higher M½ than both of the other enlarged vesicles and also have a greater maturation time in total with M_{end} at 15.0 min, 2 min greater than what is observed in the invariant chain enlarged vesicles.

4.4. Rab5-wt-mCherry Dynamics, During Maturation In Cells Expressing Rab7-wt-EGFP

Rab7-wt was introduced into the various systems to see if the increased expression would have any effect on the dynamics of Rab5 during maturation. To investigate these possible influences of Rab7, MDCK cells were transiently transfected cells with both Rab5-wt-mCherry and Rab7-wt-EGFP. In Figure 4.4-2, maturation has been visualized in all three systems of creating enlarged vesicles. Additionally to maturation analysis (Figure 4.4-3), measurements of both co-localization and distribution were performed (Figure 4.4-1 A & B respectively) to investigate if Rab7 had any effect on these. The maturation data was collected with a 30 sec interval between frames (Movie 5, Movie 6 and Movie7) and the data was analyzed in ImageJ and these data are presented in Figure 4.4-3.

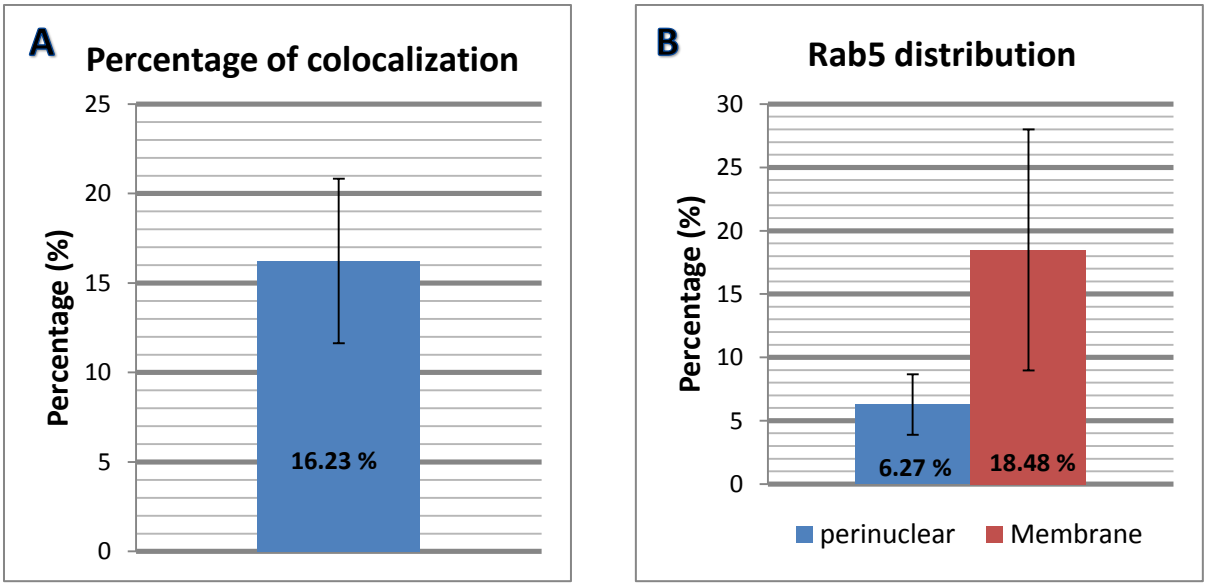

FIGURE 4.4-1: CO-LOCALIZATION AND DISTRIBUTION MEASUERED FOR CELLS TRANSIENTLY TRANSFECTED WITH RAB5-WT-MCHERRY AND RAB7-WT-EGFP. A) THE PERCENTAGE OF ENDOCYTIC COMPARTMENTS POSITIVE FOR BOTH RAB5 AND RAB7 WERE CALCULATED TO BE 16.23 % ± 4.2%. (N = 10) B) THE PERCENTAGE OF RAB5 POSITIVE ENDOCYTIC COMPARTMENTS LOCATED CLOSE TO THE NUCLEUS OR PM. 6.27 % ± 2.3 % OF EES WERE LOCATED TO THE NUCLEUS AND 18.48 % ± 9.0 % WERE LOCATED TO THE PM. (N = 10)

The results presented in 4.4-1 provide a good foundation for detecting any impact the Rab7 mutants might have on Rab5 in the cell.

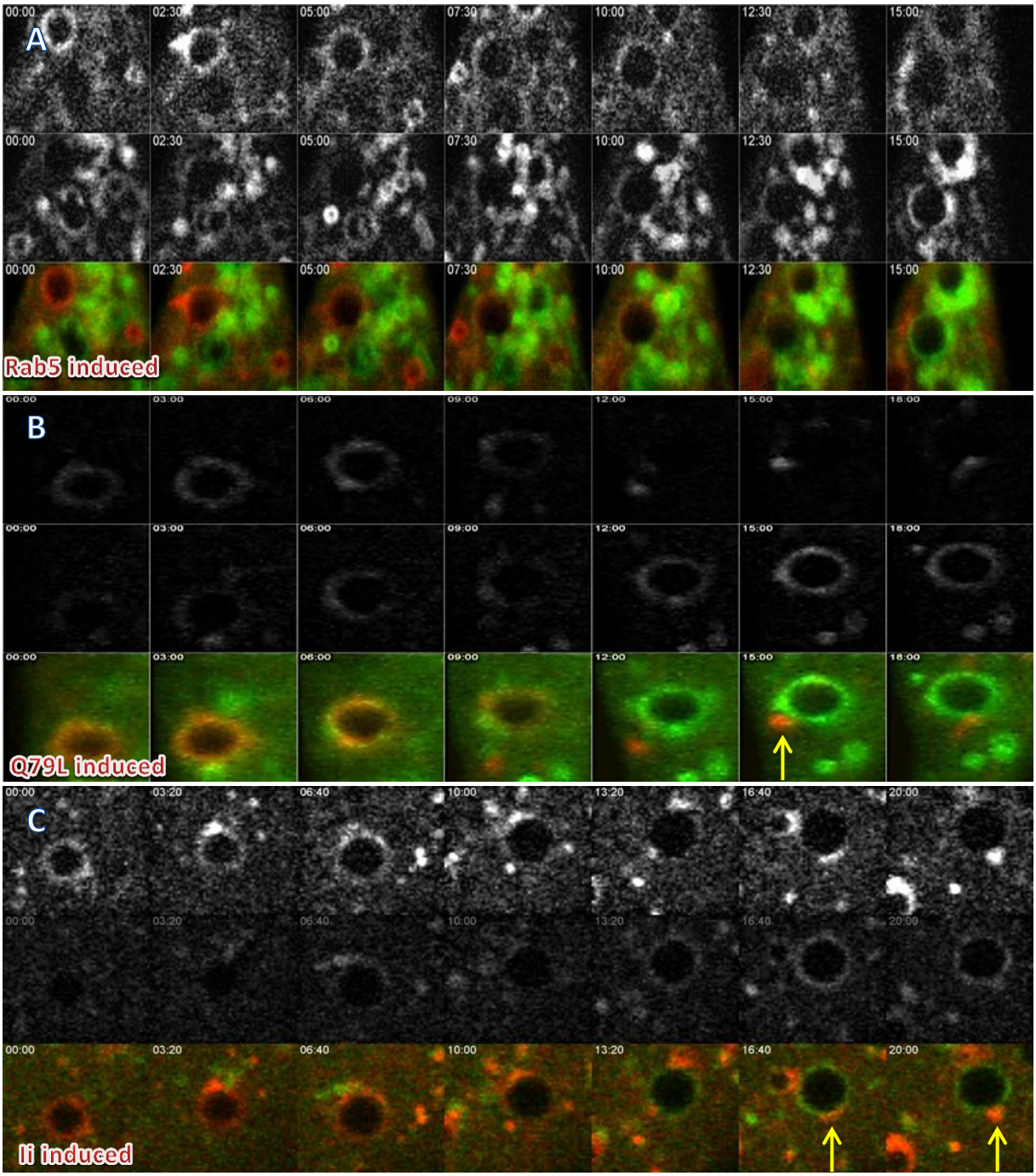

FIGURE 4.4-2: MATURATION OF SINGLE ENDOSOMES IN ALL 3 SYSTEMS. A) RAB5-WT-MCHERRY (TOP ROW) AND RAB7-WT-EGFP (MIDDLE ROW) IN A RAB5-WT-INDUCED SYSTEM. MERGED CHANNELS IN BOTTOM ROW. B) RAB5-WT-MCHERRY (TOP ROW) AND RAB7-WT-EGFP (MIDDLE ROW) IN A RAB5-Q79L-INDUCED SYSTEM. MERGED CHANNELS IN BOTTOM ROW. C) RAB5-WT-MCHERRY (TOP ROW) AND RAB7-WT-EGFP (MIDDLE ROW) IN AN II-INDUCED SYSTEM. MERGED CHANNELS IN BOTTOM ROW. IN BOTH IMAGE SERIES B & C THE LATE INTERACTION BETWEEN THE NEWLY FORMED LE AND A RAB5 POSITVE VESICLES CAN BE SEEN (ARROWS). IT IS LIKELY THAT IT IS BUDDING OF WITH THE LAST REMAINING RAB5-GTP (DISCUSSED IN SECTION 4.7)

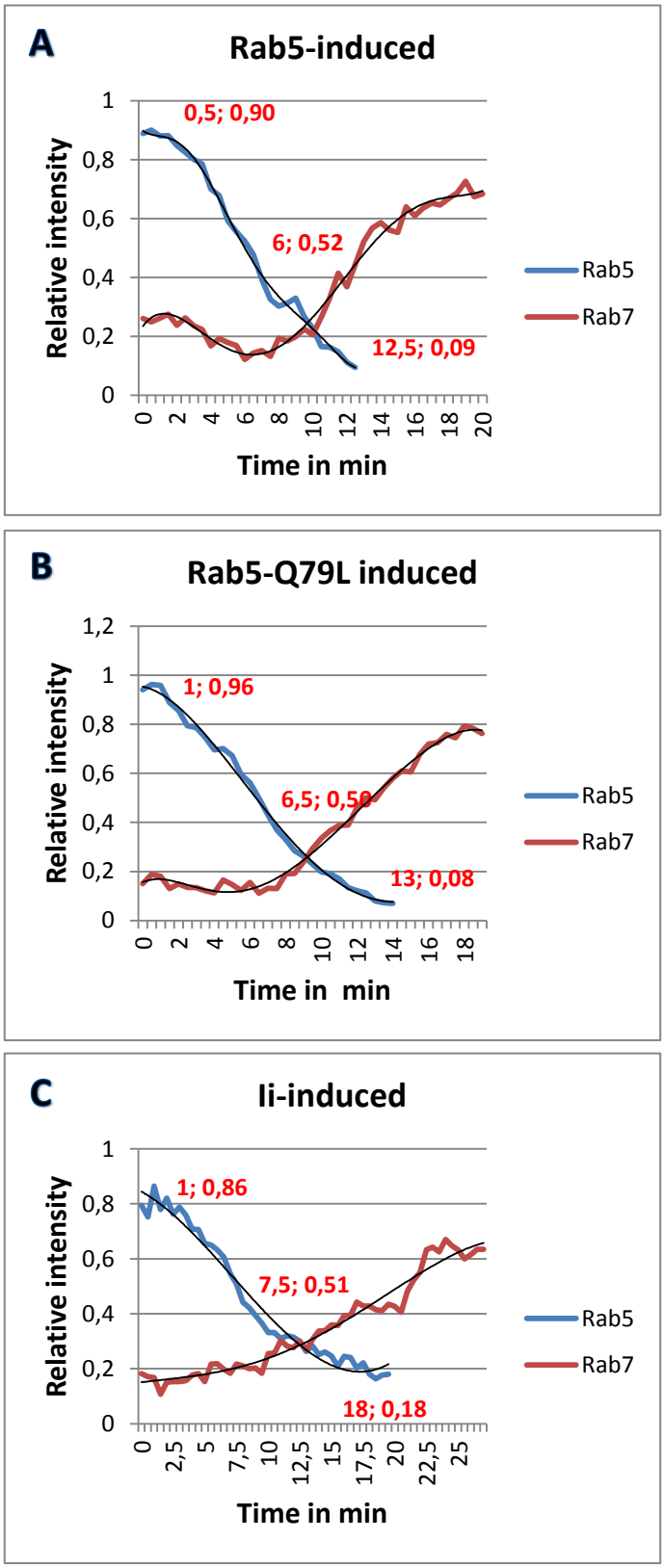

FIGURE 4.4-3: DATA SHOWING THE DECLINE OF RELATIVE INTENSITY OF RAB5-WT AND INCREASE OF RAB7-WT IN CELLS TRANSIENTLY TRANSFECTED WITH BOTH RAB5-WT-MCHERRY AND RAB7-WT-GFP. ALL DATA ARE SYNCHRONIZED AROUND THE DATA POINT FOR RAB5 CLOSEST TO 0.5 RELATIVE INTENSITY (RI) A)) RAB5-WT ENLARGED VESICLES (N = 13), M_{START}=0.5 MIN/ 0.90 RI, M½= 6.0 MIN ± 1.5 MIN/ 0.52 RI, AND M_{END}=12.5 MIN ± 0.5 MIN/ 0.09 RI . B) RAB5-Q79L ENLARGED VESICLES (N = 9), M_{START}=1.0 MIN/0.96 RI, M½= 6.5 MIN ± 1.4 MIN/ 0.50 RI, AND M_{END}=13.0 MIN ± 1.3 MIN/ 0.08 RI . C) II INDUCED ENLARGED VESICLES (N = 10), M_{START}=1.0 MIN/0.86 RI, M½= 7.5 MIN ± 0.7 MIN/ 0.51 RI, AND M_{END}=18.0 MIN ± 2.2 MIN/ 0.18 RI

The introduction of Rab7-wt-EGFP, which has been transiently transfected into the cell along with Rab5-wt-mCherry, gives a set of results diverging from the results in Table 4.3-1.

Table 4.4-1	**M½**	**M_{END}**
Rab5 induced	**5,5 min ± 1.5 min**	**12,0 min ± 0.5 min**
Rab5-Q79L-induced	**5,5 min ± 1.4 min**	**12,0 min ± 1.3 min**
Ii-induced	**6,5 min ± 0.7 min**	**17,0 min ± 2.2 min**

In Rab5-wt induced vesicles, the time it takes for Rab5 to detach from the EE limiting membrane has been reduced. The former M½ at 8.5 min has been reduced with 3.0 min to 5.5 min. The M_{END} value has also been reduced with 3.0 min from 15 min to 12 min. The M½ value of the Rab5-Q79L-induced cells has increased slightly from 5.0 min to 5.5 min. However the M_{END} value has increased from 8.5 to 12.0 min. The introduction of overexpressed Rab7-wt-EGFP appear to prompt some sort of balance to the two values which differed the most when only Rab5-wt-mCherry was overexpressed, and they now mature in a similar timespan.

Furthermore, the M½ of the Ii-induced cells increased with 1 min from 5.5 min to 6.5 min. The M_{END} value also increased from 13.0 min to 17.0 min, an increase of 4 min. Another notable result from the Ii-induced system is that the overlap period where Rab5 overlaps with Rab7 is greater than those observed in the Rab5 induced systems. In the Rab5 induced systems this overlap period is about 5 min and in the Ii-induced system this is around 8.0 to 10.0 minutes. The Rab5-mCherry maturation pattern changes upon Rab7-wt-EGFP introduction in cells expressing ILEVs. This may indicate a regulatory role from Rab7-wt-GFP exerted on Rab5-mCherry during maturation.

From the graphs presented in 4.4-3 there is also an indication that the recruitment of Rab7 to the membrane during maturation seem to begin approximately around M½ in all three inducible systems.

4.5. Rab5-wt-mCherry Dynamics, During Maturation In Cells Expressing Rab7-Q67L-EGFP

Rab7-Q67L-EGFP is a mutant of Rab7, which has had its catalytic site modified in such a way that GAPs are unable to access Rab7 and return it to the inactive GDP-bound state. This results in a Rab7 mutant that is in a chronic active GTP-bound state. We introduced the dominant positive mutant Rab7-Q67L-EGFP into the three systems to further study if the altered expression of GTP-bound Rab7 had any detectable effects on dynamics of Rab5-mcherry. The maturation process seemed to proceed as normal compared to Rab7-wt transfected cells (Figure 4.5-2, Movie 8, Movie 9 and Movie 10). However, the analyzed data (Figure 4.5-3 and Table 4.5-1) exhibited a clear trend present in all three inducible systems. Both co-localization and distribution were analyzed as well, to see if Rab7-Q67L had any impact on these (Figure 4.5-1).

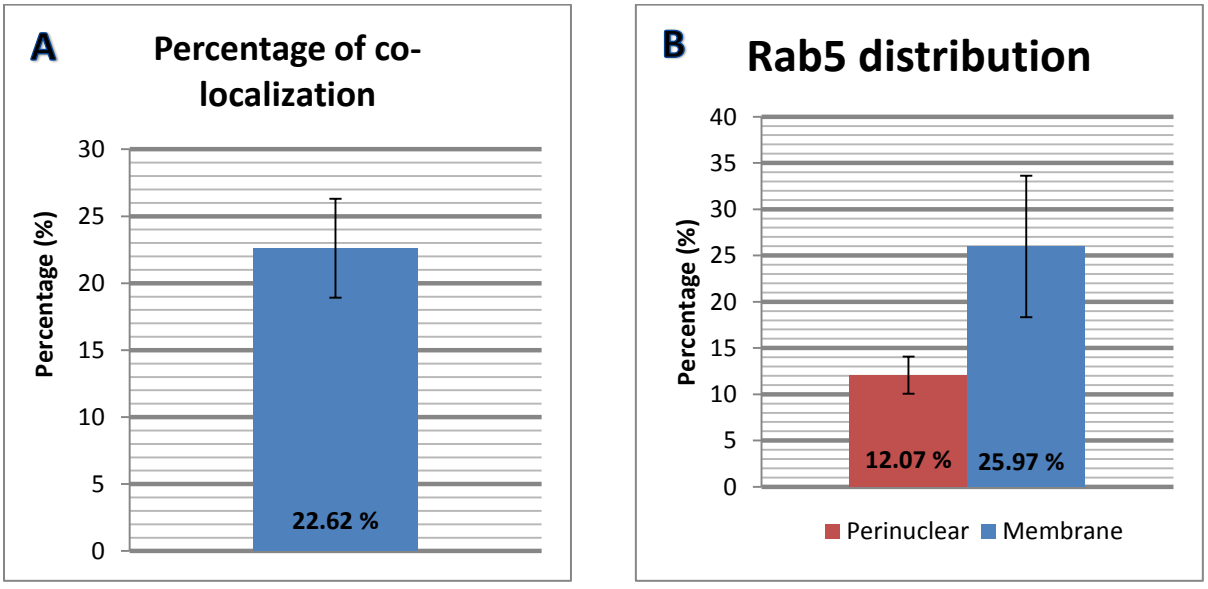

FIGURE 4.5-1: A) THE PERCENTAGE OF EES AND LES COLOCALIZING IN CELLS WITH RAB7-Q67L IS CALCULATED TO BE 22.62 % ± 3.7 %. (N = 10) B) THE PERCENTAGE OF EARLY ENDOCYTIC COMPARTMENTS THAT ARE LOCALIZED IN PROXIMITY TO THE NUCLEUS AND PM ARE 12.07 % ± 2.0 % AND 25.97 % ± 7.64 % RESPECTIVELY (N = 10)

As seen in figure 4.5-1 the percentage of co-localization is calculated to be 22.62 % ± 3.7 %, this is an increase of approximately 6.39 % compared to Rab7-wt-transfected. The distribution of Rab5 positive vesicles throughout the cells is also altered compared to Rab7-wt. In the perinuclear region the presence of Rab5 positive organelles are nearly doubled from 6.27 % to 12.07 %. Also along the PM the numbers have increased from 18.48 % in

Rab7-wt cells to 25.97 % in Rab7-Q67L cells. These results show good indications of Rab7 having a role in the positioning of vesicular Rab5-wt-mCherry compartments in the cell.

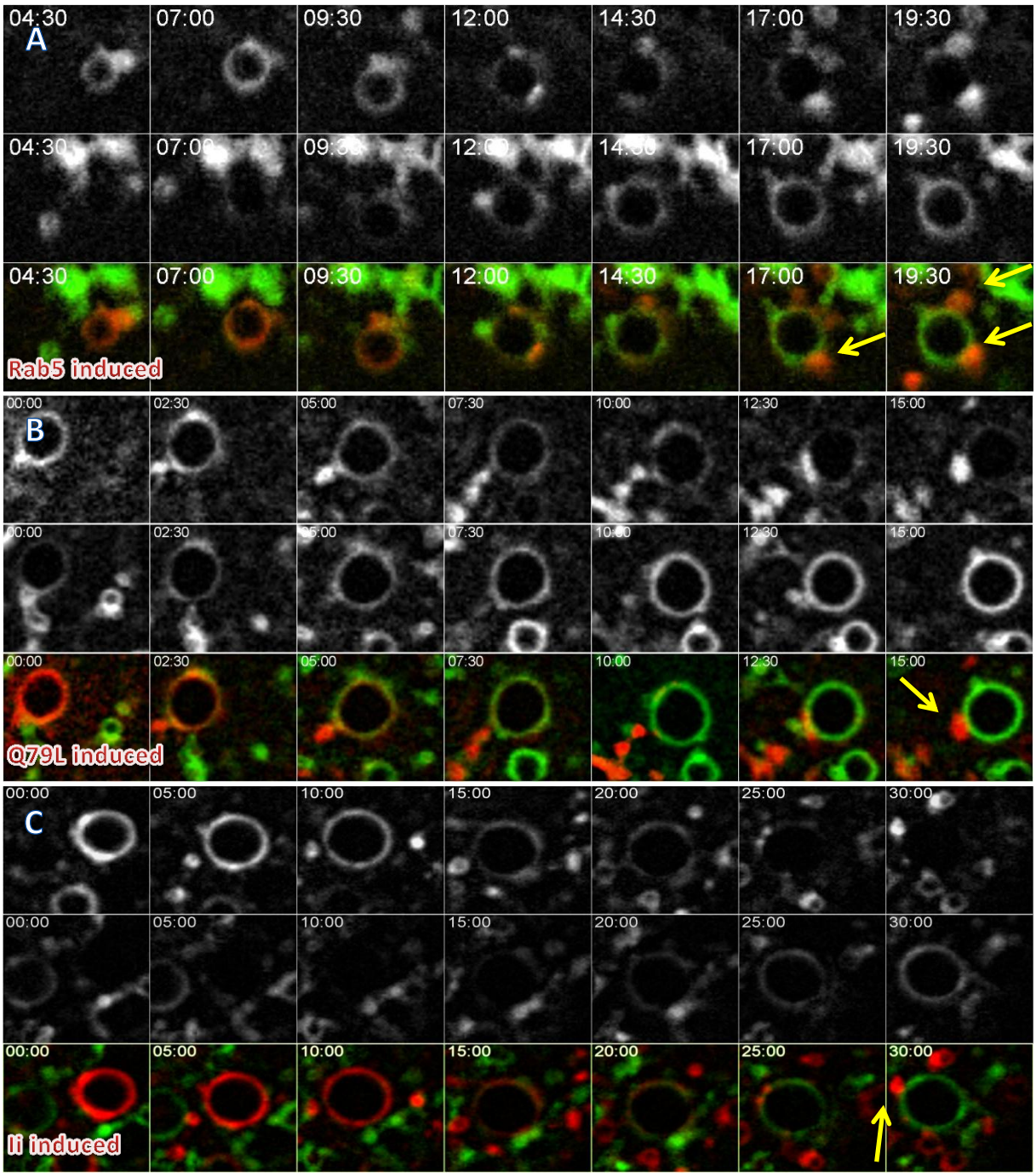

FIGURE 4.5-2: MATURATION OF SINGLE ENDOSOMES IN ALL 3 SYSTEMS. A) RAB5-WT-MCHERRY (TOP ROW) AND RAB7-Q67L-EGFP (MIDDLE ROW) IN A RAB5-WT-INDUCED SYSTEM. MERGED CHANNELS IN BOTTOM ROW. B) RAB5-WT-MCHERRY (TOP ROW) AND RAB7-Q67L-EGFP (MIDDLE ROW) IN A RAB5-Q79L-INDUCED SYSTEM. MERGED CHANNELS IN BOTTOM ROW. C) RAB5-WT-MCHERRY (TOP ROW) AND RAB7-Q67L-EGFP (MIDDLE ROW) IN A II-INDUCED SYSTEM. MERGED CHANNELS IN BOTTOM ROW. AGAIN ONE CAN SEE, IN ALL THREE IMAGE SERIES, THE RAB5 POSITIVE VESICLE (ARROWS) AT THE LES BUDDING OFF AT THE END (DISCUSSED IN SECTION 4.7). ALL THREE IMAGE SERIES ALSO SHOW AN INCREASE OF THE SIZE OF THE MATURING ENDOSOMES THAT HAS BEEN OBSERVED, BUT NOT QUANTIFIED.

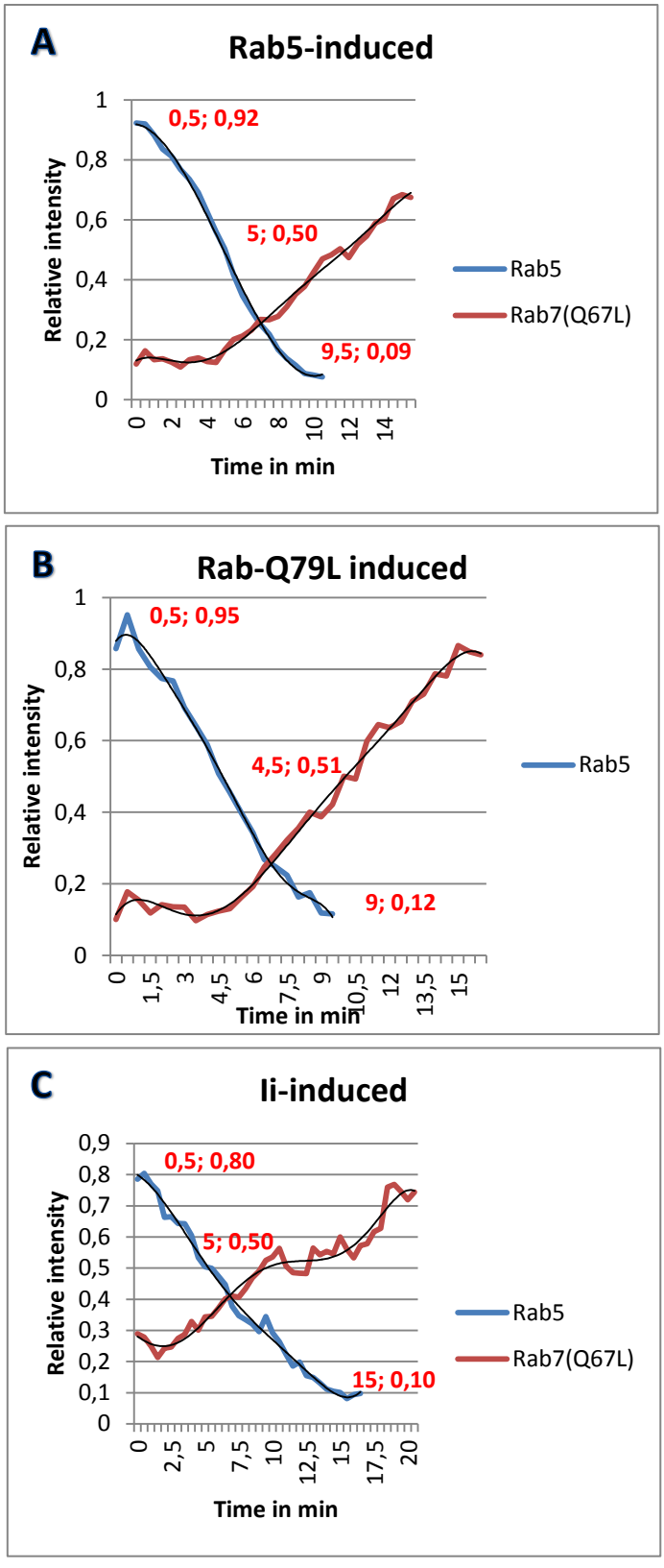

FIGURE 4.5-3: DATA SHOWING THE DECLINE OF RELATIVE INTENSITY OF RAB5-WT AND INCREASE OF RAB7-WT IN CELLS TRANSIENTLY TRANSFECTED WITH BOTH RAB5-WT-MCHERRY AND RAB7-Q67L-EGFP. ALL DATA IS SYNCHRONIZED AROUND THE DATA POINT FOR RAB5 CLOSEST TO 0.5 RELATIVE INTENSITY (RI) A) RRAB5-WT ENLARGED VESICLES (N = 8), M_{START}= 0.5 MIN/ 0.92 RI, M½= 5.0 MIN ± 0.8 MIN/ 0.50 RI, AND M_{END}= 9.5 MIN ± 0.5 MIN/ 0.09 RI . B) RAB5-Q79L ENLARGED VESICLES (N = 9), M_{START}= 0.5 MIN/ 0.96 RI, M½= 4.5 MIN ± 0.5 MIN/ 0.50 RI, AND M_{END}= 9.0 MIN ± 0.4/ 0.12 RI . C) ILEVS (N = 12), M_{START}= 0.5 MIN/ 0.80 RI, M½= 5.0 MIN ± 0.5 MIN/ 0.50 RI, AND M_{END}= 15.0 MIN ± 1.3 MIN / 0.10 RI

Rab7-Q67L-EGFP has had its GDF catalytic site inactivated by a point-specific mutation. This leaves it in a perpetual active GTP-bound state.

Table 4.5-1	M½	M_{END}
Rab5 induced	**4,5 min ± 0.8 min**	**9,0 min ± 0.5 min**
Rab5-Q79L-induced	**4,0 min ± 0.5 min**	**8,5 min ± 0.4 min**
Ii-induced	**4,5 min ± 0.5 min**	**14,5 min ± 1.3 min**

The M½ values are reduced for all experiments. The Rab5-wt-induced vesicle's M½ is reduced from 5.5 min to 4.5 min; whereas the Rab5-Q79L-induced M½ is reduced from 5.5 min to 4.0 min. For the Ii-induced vesicles the M½ is reduced with 2 min from 6.5 min to 4.5 min. That is the same M½ that is observed in the Rab5 induced vesicles. The M_{END} values are reduced from 12.0 min to 9.0 min in Rab5 induced vesicles, 12.0 min to 8.5 min in Rab5-Q79L-GFP induced vesicles, and 17.0 min to 14.5 min in Ii-induced vesicles. These results indicate that the expression of the dominant positive Rab7 can be related to an increase of the speed of maturation, and that GTP bound Rab7 in general might have an effect on the dynamics of Rab5 during maturation.

As detected in the cells transfected with Rab5-wt-mCherry and Rab7-wt-EGFP, the extended period of overlap between Rab5 and Rab7 is observed in ILEVs. In the Rab5 induced systems this overlap period is unaltered and lasts about 5.0 to 6.0 min, whereas in the Ii-induced system the period is notably longer, lasting approximately 13.0 min. For the Rab5 induced systems this is roughly the same as observed in cells expressing Rab7-wt-EGFP. On the other hand, for the Ii-induced system there is an increase of approximately 4.0 min.

Notably, in cells expressing the inducible Rab5-wt and Rab5-Q79L, the endosomal recruitment of Rab7 seems to begin at approximately M½, however, for in the Ii-induced system we could observe an earlier recruitment coinciding with M_{start}. This may be the effect of a mutual feedback loop between Rab5 and Rab7.

4.6. Rab5-wt-mCherry Dynamics, During Maturation In Cells Expressing Rab7-T22N-EGFP

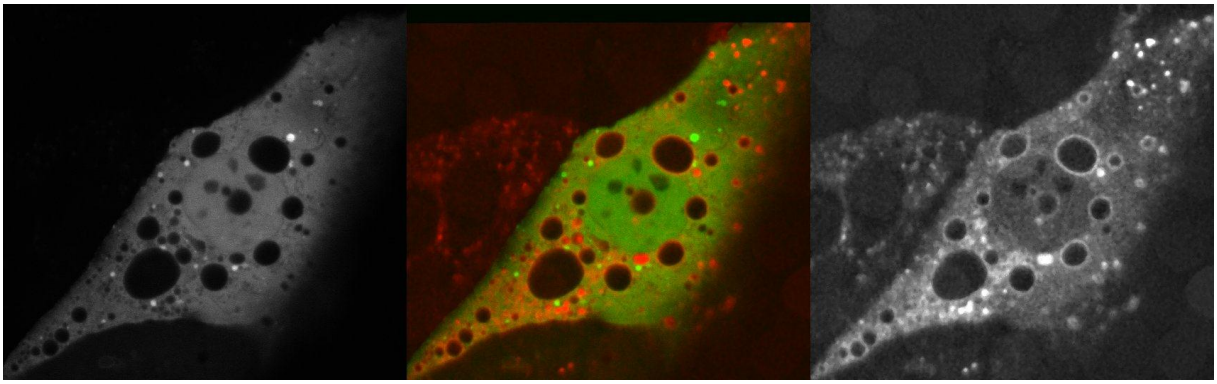

FIGURE 4.6-1: THE APPEARANCE OF A CELL TRANSFECTED WITH RAB7-T22N-GFP (GREEN) AND RAB5-WT-MCHERRY (RED) IN AN II INDUCED CELL. RAB7-T22N IS INCAPABLE TO BIND GTP RESULTS IN A PERMANENT CYTOSOLIC GDP-BOUND STATE.

Rab7-T22N-GFP has had its catalytic site modified in such a way that it is no longer able to bind GTP. It is therefore bound in its inactive GDP-bound state. This results in a Rab7 mutant that is unable to bind to the membranes of LEs, and is confined to its cytosolic state (Figure 4.6-1). Co-localization is therefore not an issue; however, the distribution of Rab5 positive vesicles is still possible to calculate in order to see if Rab7-T22N has any apparent effects on the localization. This dominant negative Rab7 ought to be a nice tool to investigate the kinetics of Rab5 in a lack of presence of Rab7-GTP during maturation as well. Considering maturation, one of the most apparent effects of the introduction of Rab7-T22N is the dramatic decrease of endocytic compartments maturing. Some EEs might begin maturing, sometimes losing most of their Rab5 coat, but somehow terminate the maturation process and return to the normal EE characteristics in terms of Rab5-wt-mCherry intensity (Movie 11). This is observed in all inducible systems, but in the Rab5-induced systems, a few complete maturations are observed (Figure 4.6-3, Movie 12 and Movie 13). These maturations indicated quite extreme alterations in the dynamics of Rab5 compared to the two other variants of Rab7, as shown in figure 4.6-4 and Table 4.6-1.

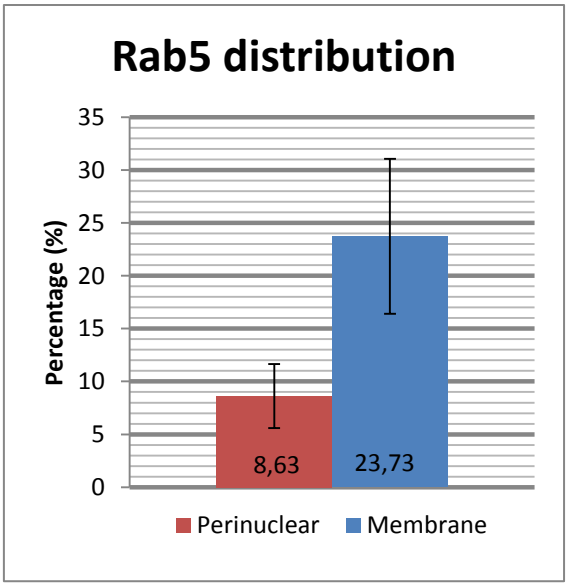

FIGURE 4.6-2: THE DISTRIBUTION IN CELLS TRANSFECTED WITH THE DOMINANT NEGATIVE MUTANT RAB7-T22N-GFP SHOWS A LOW INCREASE IN RAB5 POSTIVE COMPARTMENTS AROUND BOTH NUCLEUS AND PM COMPARED TO THOSE TRANSFECTED WITH RAB7-WT-EGFP. WITH 8.63 % ± 3.02 % LOCATED CLOSE TO THE NUCLEUS, AND 23.73 % ± 7.33 % LOCATED TO THE PROXIMITY OF THE PM (N = 10)

The distribution of Rab5 positive vesicles in cells expressing the dominant negative mutant Rab7-T22N-GFP is calculated to 8.63 % ± 3.02 % located to the nucleus, and 23.73 % ± 7.33 % located to the PM. Compared to the distribution results from the Rab7-wt transfected cells, we observe an increase at both locations. In the perinuclear zone, the number of Rab5 positive vesicles has increased from 6.27 % ± 2.3 % to 8.63 %, an increase of approximately 2.36 %. Along the PM the number of Rab5 positive vesicles has increased from 18.48 % ± 9.0 to 23.73 % ± 7.33 %. The expected deviations found in these cells suggest that there is little or no effect exerted by Rab7-T22N-EGFP on the localization of Rab5 positive vesicles.

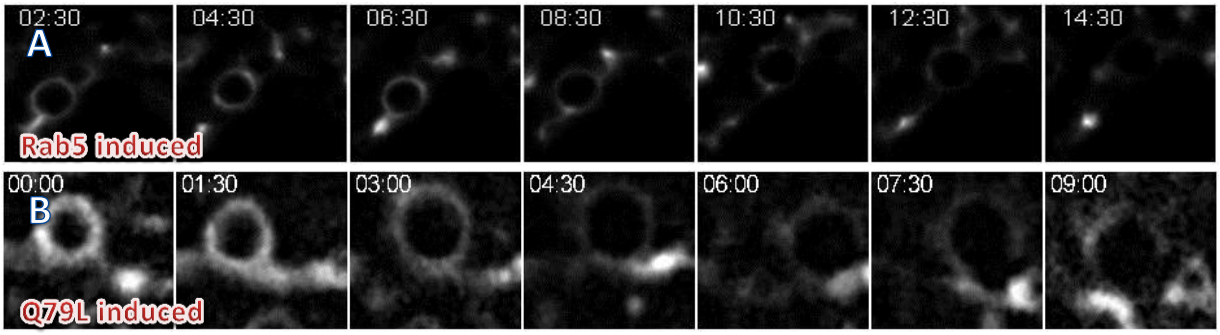

FIGURE 4.6-3: MATURATION OF A SINGLE ENDOSOME IN A) RAB5-WT-INDUCED CELLS AND B) IN Q79L INDUCED CELLS. HERE ONE CAN SEE HOW ONE OF THE FEW AND HOW THE MATURATION PROCEEDED WHEN THE CELL IS INTRODUCED TO THE RAB7-T22N-GFP MUTANT.

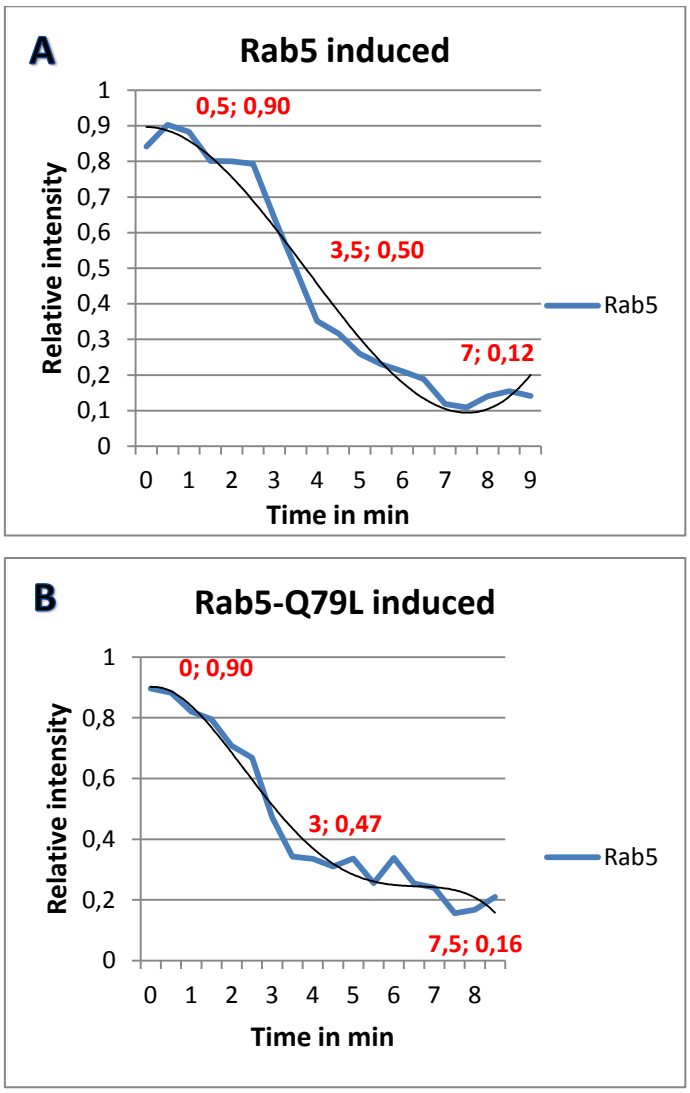

FIGURE 4.6-4: DATA SHOWING THE DECLINE OF RELATIVE INTENSITY OF RAB5-WT AND INCREASE OF RAB7-WT IN CELLS TRANSIENTLY TRANSFECTED WITH BOTH RAB5-WT-MCHERRY AND RAB7-T22N-GFP. ALL DATA ARE SYNCHRONIZED AROUND THE DATA POINTS FOR RAB5 CLOSEST TO 0.5 RELATIVE INTENSITY (RI) A) RAB5-WT ENLARGED VESICLES (N = 5), M_{START}= 0.5 MIN/ 0.90 RI, M½= 3.5 MIN ± 0.9 MIN/ 0.5 RI, AND M_{END}= 7.0 MIN ± 1.1 MIN/ 0.12 RI . B) RAB5-Q79L ENLARGED VESICLES (N = 4), M_{START}= 0.0 MIN/ 0.90 RI, M½= 3.0 MIN ± 0.7 MIN/ 0.47 RI, AND M_{END}=7.5 MIN ± 0.3 MIN/ 0.31 RI.

The dominant negative mutant Rab7-T22N-GFP has had its catalytic site mutated in such a manner that it is not able to switch to the active GFP-bound state. The numbers of EEs that mature are dramatically reduced compared any of the other experiments. EEs that remained Rab5 positive during the duration of the experiments were abundant, and many EEs that seemed to mature were not preferable candidates for analysis due to excessive motility, moving them out of focus. Some eligible EEs were, however, found as seen in Figure 4.6-3

and they showed some perplexing results. In the Ii-induced system the maturation and Rab5-wt-mCherry was too unstable to collect data suitable for analyses.

Table 4.6-1	M½	M_{END}
Rab5-induced	3,0 min ± 0.9 min	6,5 min ± 1.1 min
Rab5-Q79L-induced	3,0 min ± 0.7 min	7,5 min ± 0.3 min
Ii-induced	-	-

The M½ value for both the Rab5 and Rab5-Q79L-induced vesicles are as low as 3.0 min. The M_{end} value for the Rab5 induced system is at 6.5 minutes. In the Rab5-Q79L-induced system the M_{end} value is 7.5 minutes. In both Rab5 induced systems we observe the swiftest maturation among all the cells transfected with a variant of Rab7. However, the most remarkable result was the lack of stable maturation that may signify the lack of some regulatory control, that in this case it the active form of Rab7.

Table 4.6-2	Rab5 induced		Q79L induced		Ii-induced	
	M½	M_{END}	M½	M_{END}	M½	M_{END}
-	6,5± 1,6 min	13,0 ± 1,1 min	5,0 ± 1,3 min	8,5 ± 1,1 min	5,5 ± 1,2 min	13,0 ± 0,5 min
Rab7	5,5± 1,5 min	12,0± 0,5 min	5,5 ± 1,4 min	12,0± 1,3 min	6,5 ± 0,7 min	17,0 ± 2,2 min
Rab7-Q67L	4,5± 0,8 min	9,0 ± 0,5 min	4,0 ± 0,5 min	8,5 ± 0,4 min	4,5± 0,5 min	14,5± 1,3 min
Rab7-T22N	3,0± 0,9 min	6,5 ± 1,1 min	3,0 ±0,7 min	7,5 ±0,3 min	-	-

TABELL 4.6-2: ALL VALUES OF MATURATION FROM THE THREE DIFFERENT SYSTEMS

4.7. Vesicular Interactions Contributes During Maturation

During the analysis of maturation we observed a series of incoming and outgoing vesicles (Figure 4.7-1 and Figure 4.7-2) that interacts with the maturing EE. These are either Rab5 positive or Rab7 positive and seem to play an important role in the maturation process.

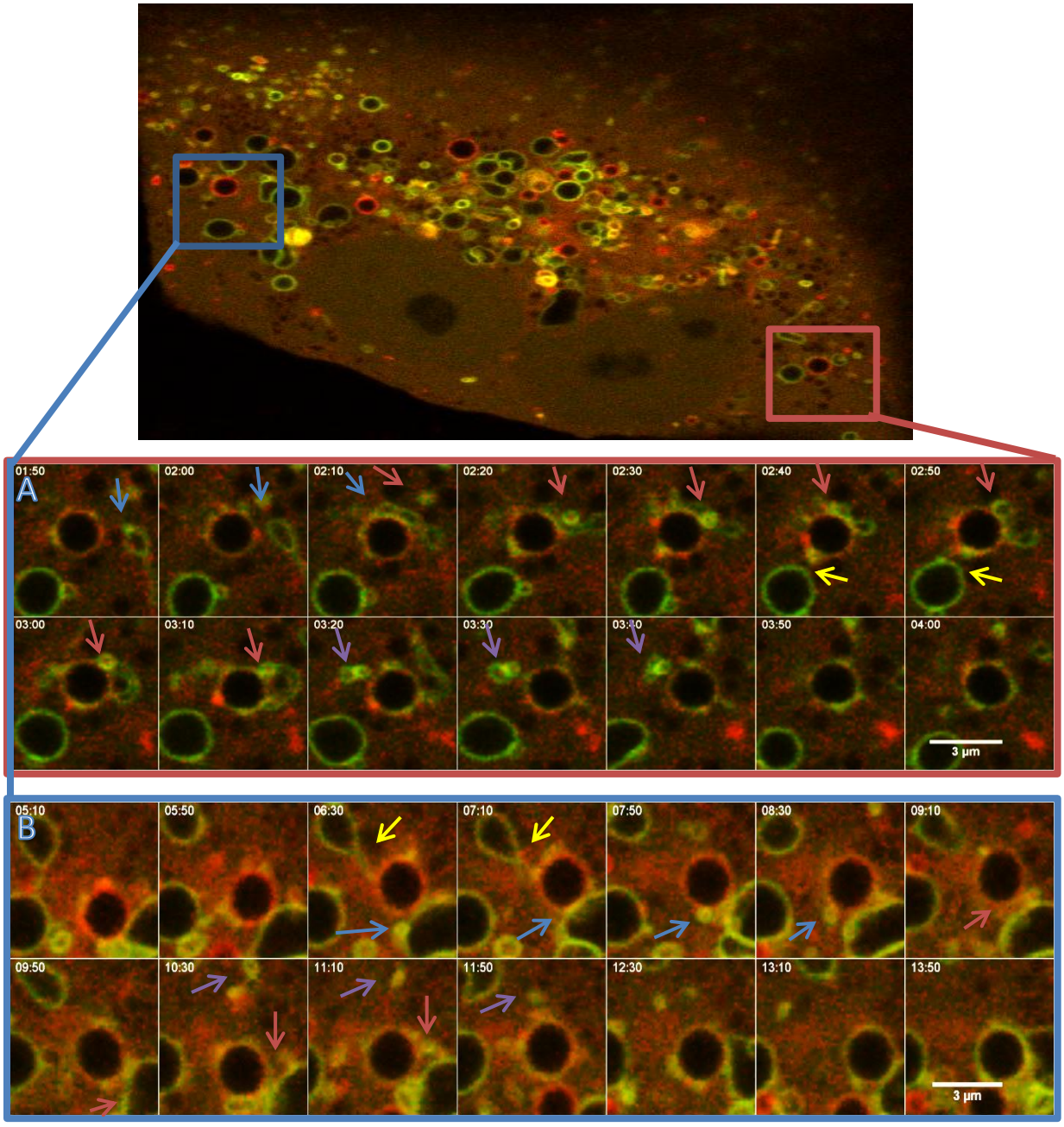

FIGURE 4.7-1: INCOMING RAB7 POSTIVE VESICLES IN A CELL TRANSIENTLY TRANSFECTED WITH RAB5-WT-MCHERRY AND RAB7-WT-EGFP IN AN II-INDUCED SYSTEM. A) RAB7 POSITIVE VESICLES INTERACT WITH THE MATURING EE OVER A SHORT TIMESPAN DURING MATURATION. THE ARROWS INDICATE POSSIBLE VESICULAR INTERACTIONS. B) RAB7 POSITIVE VESICLES INTERACT WITH THE MATURING ENDOSOME DURING A TIMESPAN COVERING MOST OF THE PROCESS OF MATURATION.

The incoming Rab7 positive vesicles seem to be providing the maturing vesicle with Rab7 (Figure 4.7-1), and possibly also a portion of their membrane. This would also explain the suspected growth during maturation. These incoming Rab7 vesicles also seem to interact with specific Rab7 domains already located on the EEs (Movie 14). These observations, however, indicate that vesicular cooperation might be necessary for an efficient maturation process.

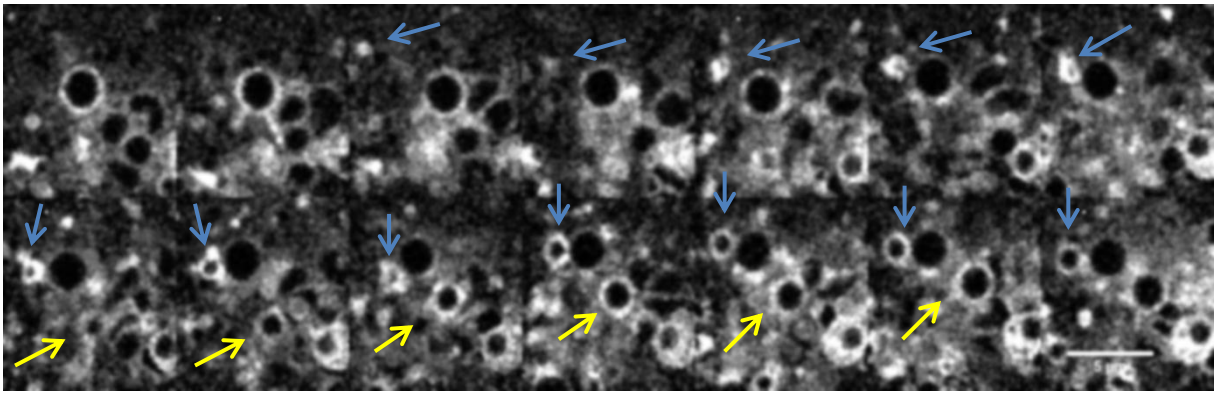

FIGURE 4.7-2: RAB5 POSITIVE VESICLE INTERACTING WITH A MATURING ENDOSOME. THE ARROWS INDICATE TWO DIFFERENT RAB5 POSITIVE VESICLES THAT APPEAR TO BE INTERACTING WITH THE MATURING ENDOSOME

Rab5 positive vesicles seem to interact with maturing endosomes. As seen in Figure 4.7-2, there is an up regulation of Rab5 positive activity during maturation compared to earlier. The Rab5 positive vesicles that interact with the maturing vesicle seem to grow in size; however, it is unclear if this is due to their interaction with the maturing endosome and not a consequence of homotypic fusion. It is also possible to assume that the incoming Rab5 positive vesicles might grab a portion of the Rab5 on the membrane of the maturing endosome.

In Figure 4.4-2 and Figure 4.5-2 it also showed that what appears to be a fully matured LE either has a Rab5 positive domain or a closely associated Rab5 positive vesicle. These data are not quantified and possible purposes for these vesicles are not known. They can be a result of remaining Rab5, which cluster together towards the very end of maturation, and bud of generating an EE.

4.8. FRAP results

Calculations of the maturation dynamics indicated a communication between Rab5 and Rab7 during maturation. It has been reported several times that the maturation is controlled by the coat kinetics of the two respective proteins (Rink, Ghigo et al. 2005) and according to previous results, a positive/negative feedback loop between these may be in control of the coat kinetics. If Rab7 exert some kind of positive feedback on Rab5 during maturation Rab7 might also control the Rab5-coat kinetic prior to maturation.

We asked ourselves the question; does Rab7 exert any control of the Rab5-coat on-off cycling? To figure this out we bleached Ii enlarged, Rab5-mCherry positive endosomes in cells expressing either Rab7Q67L or Rab7T22N.

As a control experiment, Rab5-wt-mCherry was bleached in cells expressing Ii-chain enlarged endosomes (4.8-1). In the control experiment we measured the T½ recovery to be 20.9 ± 1.9 seconds (4.8-2). Similar experiment with additionally T22N and Q67L transfected were performed to see characterize any change in Rab5-wt-mCherry coat kinetics.

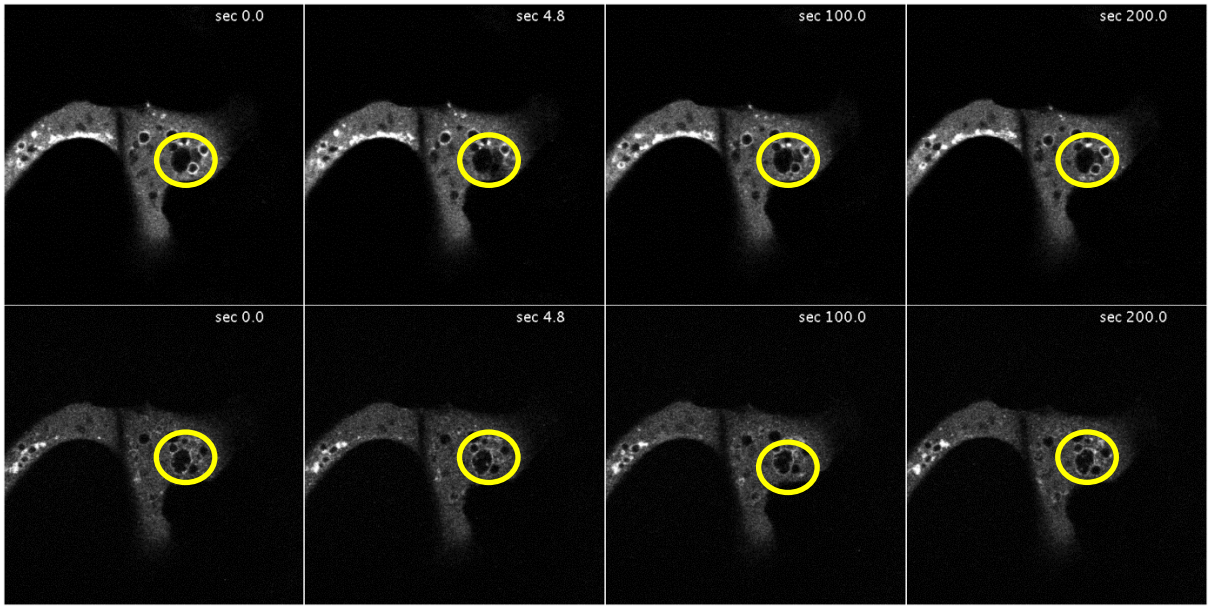

FIGURE 4.8-1: MDCK CELLS WERE TRANSIENTLY TRANSFECETED TO EXPRESS RAB5-WT-MCHERRY AND RA7-Q67L-GFP. II WAS USED TO GENERATE ENLARGED VESICLES. STABLE EARLY ENDOSOMES WERE TARGETED WITH THE 559 SOLID STATE LASER AT 100 % INTENSITY FOR 1000 µS. THIS RESULTED IN BLEACHING FOR RAB5-WT-MCHERRY. THE RECOVERY OF RAB5 WAS THEN MEASURED ACCORDINGLY.

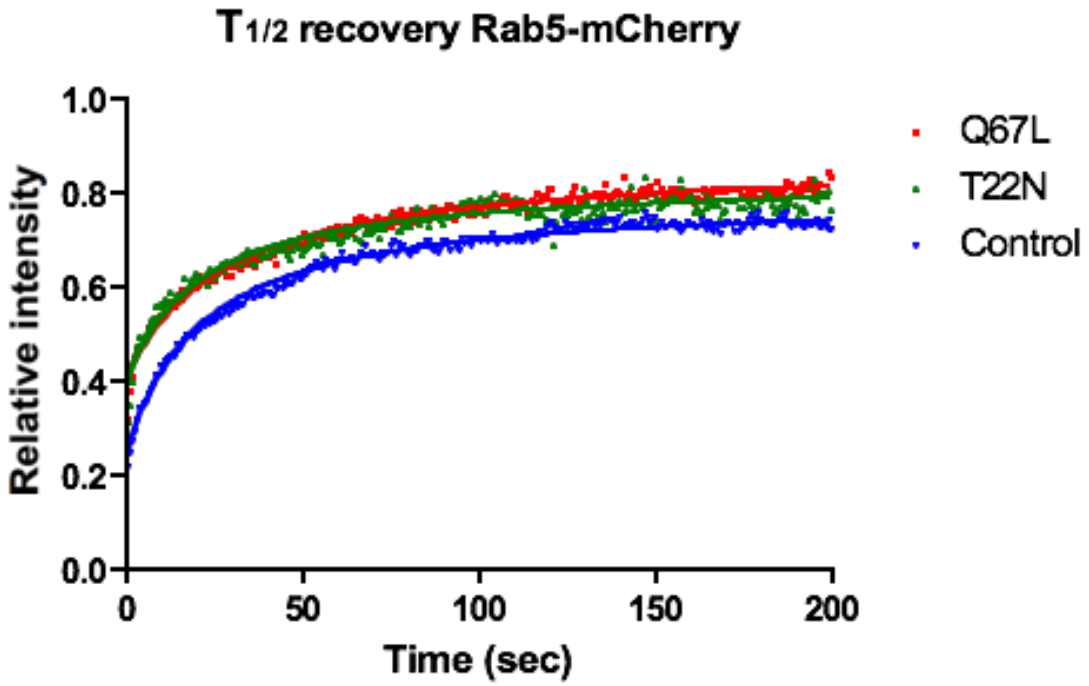

FIGURE 4.8-3: FRAP CURVES REPRESENTING T½ RECOVERY FOR RAB5-MCHERRY IN SECONDS. Q67L N=10, T22N N=6, CONTROL N=14

In cells transiently transfected with both Rab5-wt-mCherry and Rab7-T22N-GFP we measured the T½ recovery to be 22.3 ± 2.0 seconds which is reminiscent to the control experiment. However, in cells expressing Q67L we could calculate an increase in T½ to 27.8 ± 2.2 seconds recovery for Rab5-wt-mCherry. This result is a good indication that there is a directed feedback from Rab7 to Rab5 controlling the kinetics and may represent an important regulatory role in Rab5-wt-mCherry on-off kinetics and endosomal maturation.

5. Discussions

By utilizing the fusogenic properties of Rab5 and Ii, we have been able to investigate the dynamics of coat proteins residing on EEs and maturing EEs. The enlarged endocytic compartments properties such as the size and reduced motility has been shown to be very advantageous in cell biological studies (Bergeland, Haugen et al. 2008; Skjeldal, Strunze et al. 2012). In this study we have utilized the properties of three well-known fusogenic proteins Rab5wt, RabQ79L and Ii-chain. Rab5, one of the introduced fusogenic protein, are endogenously expressed in the cells. However, overexpression of this protein, either Rab5wt or Rab5Q79L induce enlargement of the endocytic compartments but also alters the morphology of the enlarged structures (Wegner, Malerod et al. 2010). Ii-chain is an immunological molecule known to act as a fusogenic protein (Stang and Bakke 1997; Gregers, Nordeng et al. 2003) and increase the lifetime of early endosomes (Landsverk, Barois et al. 2011). On the other hand, we believe that when introducing Ii into our model cell line, we do not interfere with the natural balance of the endogenously fusogenic machinery as with Rab5 expression.

Several studies have shown that overexpression of Rab7 and different mutants induce a change in the late endosomal/lysosomal localization (Bucci, Thomsen et al. 2000). Moreover, an induced change in either Rab5 or Rab7 expression can additionally change general co-localization of the respective proteins on endosomes. We performed both co-localization and localization analysis of Rab5-wt-mCherry positive vesicles in cells expressing enlarged vesicles co-transfected with Rab7. There are several methods for calculating co-localization and calculating this by overlapping pixels seem to be to most extensively used method. However, as specialized coat proteins are not found uniformly on the membrane on EEs but in micro-domains, which do not necessarily physically overlap (Sonnichsen, De Renzis et al. 2000); we made use of a method defining the EE in as a ROI and calculate how many of these ROIs who also had an expression of Rab7 within (See materials and methods Section 3.6.3). By using this method we calculated the co-localization to be 16.23 % ± 4.2 % (Figure 4.4-1 A), reminiscent to a previous study where co-localization was calculated to be 14.6 % between Rab5-wt and Rab7-wt (Vonderheit and Helenius 2005). In cells were we had co-transfected

with both Rab5-wt-mCherry and Rab7-Q67L-EGFP, co-localization results indicated a larger percentage of endosomal compartments now overlapping Rab7 with Rab5 (Figure 4.5-1 A). This might be because Rab7-Q67L-EGFP can be able to bind to early endosomal membranes and form stable Rab7 domains more effortlessly. Or it could be that Rab7 domains are more stable as a consequence of the dominant positive Rab7. Furthermore, this increased co-localization might affect the coat dynamics of Rab5.

The localization of Rab5 positive vesicles were calculated by defining a region both in the perinuclear region and along the plasma membrane. In cells transfected with both Rab5-wt-mCherry and the different forms of Rab7, localization data was collected (Figure 4.4-1 B, Figure 4.5-1 B, and Figure 4.6-1). Along the plasma membrane the localization of Rab5-wt-mCherry positive endosomes were similar, identifying approximately 20-25 % of Rab5-wt-mCherry positive vesicles in this region. In the cells expressing Rab7-Q67L-EGFP (Figure 4.5-1 B) the calculated number of Rab5-positive vesicles localized in the perinuclear region was seemingly higher compared to the other experiments (Figure 4.4-1 B and Figure 4.6-1). The reasons behind this tendency are fairly unclear; however, it implicates Rab7, through regulation of Rab5 dynamics, may control the localization of EEs.

From the co-localization and localization study of Rab5-mCherry positive endosomes study we could observe a change in cells co-expressing Rab7. This indicated some kind of Rab7 regulation of the Rab5-mCherry positive EE localization and the co-localization between the two individual proteins. We wanted further to analyze if the increased expression of Rab7-wt-EGFP and the mutants could induce a change in the rab5-mCherry coat dynamics during maturation. In Figure 4.3-2 we presented the data for the detachment off Rab5 during maturation in cells transfected with Rab5-wt-mCherry in the various inducible systems. The M ½ values that were presented were 6.5 min ± 1.6 min for the Rab5 induced cells, 5.0 min ± 1.3 min for the Rab5-Q79L-induced system, and 5.5 min ± 1.2 min for the Ii-induced system. The M_{end} values for these systems were 13.0 min ± 1.1 min, 8.5 min ± 1.1 min and 13.0 min ± 0.5 min, for the Rab5-wt-induced, Rab5-Q79L-induced, and the Ii-induced cells respectively. With the StdDev overlapping in the results for M½, the three different cellular systems seem to represent a similar maturation pattern, analyzed by a loss of Rab5-wt-mCherry coat. As EE maturation is a highly dynamic process, and as there might be local circumstances that have an impact upon the results, however, the fastest Rab5-wt-mCherry detachment in total was

observed on the Rab5-Q79L-induced system. Here we have calculated a M_{end} that was 4.5 minutes lower than that of the two other systems.

In cells expressing enlarged endosomes induced by expression of Rab5Q79L there might be that Rab5-wt-mCherry has to compete for binding to early endosomal membranes due to the high expression of Rab5-Q79L. Rab5-Q79L in its persistent GTP-bound state increases the portion of the immobile fraction of Rab5 found on EE compared to cells expressing Rab5-wt (Bergeland, Haugen et al. 2008). This Rab5-Q79L immobile fraction will not disassociate from the maturing endosome and will result in increased overlapping of EE and LE markers (Figure 4.1.2-1 B). This result in a smaller fraction of Rab5-wt-mCherry that actually detach during maturation and as a consequence the detachment rate is faster.

With the maturation analysis of Rab5-wt-mCherry transfected vesicles as a control, we could now further investigate if any of the different Rab7s exercised any influence on the Rab5-wt-mCherry uncoating. Compared to preceding experiments we observed an increase in M½ in both of the Rab5-Q79L s induced system and the Ii-induced system. In the Rab5-wt-induced system the M½ was reduced from 6.5 min ± 1.6 min to 5.5 min ± 1.5 min, in the Rab5-Q79L-induced the M½ was increased from 5.0 min ± 1.3 min to 5.5 min ± 1.4 min, and in the Ii-induced system it increased from 5.5 min ± 1.2 min to 6.5 min ± 1.2 min. Again the M½ do not differ considerably. The M_{end} values also differ from earlier; The M_{end} value for the Rab5-wt system has decreased from 13.0 min ± 1.1 min to 12.0 min ± 0.5 min. In the Rab5-Q79L-induced system the M_{end} value have greatly increased from 8.5 min ± 1.1 min to 12.0 min ± 1.3 min. This increase is also observed for the M_{end} value for the Ii-induced system which has increased from 13.0 min ± 0.5 min to 17.0 min ± 2.2 min. Even though the M½ value did not represent any change in this experiment, we could detect a change in M_{end}. An increase of the M_{end} values for the Rab5-Q79L-induced system and the Ii-induced system was observed, indicative of a Rab7-wt prolonging effect on the maturation process.

Interestingly the maturation analysis in cells transiently transfected with both Rab5-wt-mCherry and Rab7-Q67L-EGFP showed a trend in all three systems. The M½ calculated for all 3 systems was reduced compared to the results attained in from the Rab7-wt experiments. In the Rab5-wt-induced system the M ½ was reduced from 5.5min ± 1.5 min to 4.5 min ± 0.8

min, in the Rab-Q79L it was reduced from 5.5 min ± 1.4 min to 4.0 min ± 0.5 min. The M_{end} values for both these systems are also reduced, while they were both at approximately 12 min they are now 9.0 min ± 0.5 min for the Rab5-wt-induced system and 8.5 min ± 0.4 min. For the Ii-induced system the M½ is reduced from 6,5min ± 0.7 min to 4.5 ± 0.5 min, and the M_{end} value has decreased from 17.0 min ± 2.2 min to 14.5 min ± 1.3 min. The point that the decrease of Rab5 intensity is reduced in such a similar way in all three systems is a good indication that Rab7-Q67L-EGFP has a regulatory function on Rab5-wt-mCherry dynamics during maturation.

In cells transiently transfected with Rab5-wt-mCherry and the dominant negative Rab7-T22N-GFP, Rab5m-Cherry coat detachment was very unstable. It was only in the Rab5 induced systems where maturation could be observed acting in a similar manner as observed in the earlier experiments (Figure 4.6-3). We could observe several partial Rab5-wt-mCherry coat detachments, and then subsequently see the vesicles regain the Rab5-wt-mCherry coat (Movie 11). The number of eligible EEs that were stable enough to be analyzed was severely reduced and this resulted in less than wanted results for quantification. However, the few we managed to analyze displayed a surprising tendency and the coat detachment was even faster than previously measured. The M½ in the Rab5-wt induced system has been reduced from the 5.5 min ± 1.5 min seen in Rab7-wt-EGFP transfected cells to 3.0 min ± 0.9, and the M_{end} value has decreased from 12.0 min ± 0.5 min to 6.5 min ± 1.1 min. The same trend is seen in Rab5-Q79L induced systems as M½ is reduced from 5.5 min ± 1.4 min to 3.0 min ± 0.7 min and the M_{end} value has decreased from 12.0 min ± 1.3 min to 7.5 min ± 0.3 min. In the Ii-induced system we found no EEs since the Rab5-mCherry dynamics were very unstable. These observations may be due to the down regulation of endogenous Rab7 when the cells are transfected with one of the Rab7 variants (Progida et al, unpublished results). However, when the cells were transfected with the Rab7-T22N-EGFP the regulation of Rab5-mCherry coat detachment seems to be impaired and this implies a Rab7 regulation of Rab5-mCherry dynamics is important during maturation. This may be an effect of the low level of endogenous and functional Rab7 as previously mentioned (Progida et al, unpublished results) , which indicates a regulatory role for Rab7 in Rab5-wt-mCherry coat control.

Data from the maturation analysis on Rab5-wt-mCherry coat dynamics provided us with indications of an individual RAb7 to Rab5 feedback control. To analyze the Rab5-mCherry coat kinetics prior to maturation in the presence of different Rab7 mutants, we turned to FRAP experiments. Similar cell lines were used and we bleached Rab5-mCherry in the presence of the different Rab7 previously mentioned.

In cells expressing Ii-induced vesicles the native fusion system involving Rab5, EEA1 and SNAREs is not influenced as with cells overexpressing Rab5 (Bergeland, Haugen et al. 2008). An overexpression of Rab5 also increases the immobile fraction on EEs (Bergeland, Haugen et al. 2008). We believe that this effect might unnecessary disturb the normal coat dynamics found on EEs compared to untreated cells. This, and based on the previous results we decided to use the Ii-induced enlarged endosomes for the bleaching experiments.

In the control experiment with cells only expressing Rab5-mCherry on Ii-chain enlarged endosomes we calculated the T½ recovery to be 20.9 ± 1.9 seconds (similar to ref?). When co-transfecting Rab7-T22N-EGFP we could not detect any specific change in the T½ recovery and it was measured to 22.3 ± 2.0 seconds. With overlapping STD this does not show any specific change in the Rab5-mCherry kinetics. However, when we co-transfected Rab7-Q67L-EGFP we could observe an interesting shift in T½ recovery compared to the control and Rab7T22N and it was calculated to 27.8 ± 2.2 seconds. This is an interesting result where we can measure a feedback mechanism from Rab7 on Rab5 in order to control the rab5 kinetics prior to maturation.

In this study we have designed a model based on florescence intensity measurement and FRAP analysis for detecting cellular factors that can impose a shift in the endosomal maturation. We have not been able to identify the mechanism behind the effect Rab7 has on Rab5 on the endosomal membrane, however, we may speculate to how Rab7 may apply this effect. During the maturation process, both Rab5 and Rab7 associate with the Rab7 GEF Mon1-Ccz1 complex (Nordmann, Cabrera et al. 2010). This complex has also been reported to disrupt the Rab5 positive feedback loop during maturation, by displacing Rabex5 (Poteryaev, Datta et al. 2010). If both Rab5 and Rab7 in their GTP-bound state are important for Mon1-Ccz1 localization to EEs, it is not unthinkable that this complex might be recruited in a larger scale to the membrane of EEs. This, additionally to the observation that Rab7-

Q67L-EGFP may occur more frequent at the limiting membrane of EEs (Figure 4.5-1 A), can then create Rab7 micro domains on EEs which disturbs the Rab5 feedback loop compared to the other Rab7s.

We also observed that the expression of Rab7-Q67L-EGFP lowered the M½ and M_{end} values in all three inducible systems compared to the experiments conducted with Rab7-wt-mCherry. In the light of the FRAP results for the same Rab7 mutant, these are quite reasonable results indicating a disruptive effect towards the binding dynamics of Rab5-wt-mCherry, and if the on-off dynamics for Rab5 is already disturbed prior to maturation, then this solely might be enough to increase the maturation rate. If the proposed interaction above, where Rab7-Q67L-EGFP recruited mon1-ccz1 complex disturbs the Rab5 feedback-loop, then an up regulated recruitment of this complex might also be expected during the maturation process. On the other side the mon1-ccz1 complex has been identified as the switch for endosomal maturation, nevertheless, this complex is not likely to solely control this, and there are several factors such as pH, PtdIns, and other protein interactions cooperating to make maturation happen.

In section 4.7 we described what appear to be specific vesicular interactions during maturation (Figure 4.7-1 and Figure 4.7-2). In Figure 4.7-1 A, one of these interactions is clearly visible. This appear to be a direct interaction which correlates with the maturation process, which raise the question if the recruitment of Rab7 to the maturing endosome is not solely from the cytosol, but also relocated from another Rab7 positive vesicle. This could imply a sort of kiss and run action, but it could also be that the Rab7-positive vesicles fuse with the maturing EE. This could explain the increase of size of the maturing EE (Data not shown). If the purpose of the incoming Rab7-positive vesicle is to drop of GTP-bound Rab7 in some way, then these interactions are likely to be important for the maturation process. It could be that the cytosolic gradient of GDP-bound Rab7 is too low for an effective maturation process to rely on the GEF capabilities of Mon1-Ccz1-complex alone. The interactions between these Rab7 positive vesicles also appear to be targeted towards already existing Rab7 micro domains on the EEs (Movie 14)

Considering the interactions seen between the maturing EEs and Rab5 positive vesicles, there is a bit more uncertainty to if these are specifically related to the maturation process.

Homotypic fusion is a frequently occurring incidence between EEs and there might be nothing to it than that. Nonetheless, there is the possibility that incoming Rab5 positive vesicles interact with maturing EEs to recycle both coat proteins and/or cargo. This is not implausible as an increase in tubule formation has been reported during maturation (van Weering, Verkade et al. 2012).

Based on the results from Rab5-mCherry coat detachment during maturation and FRAP data, Rab7 seems to impose a regulatory factor on Rab5-mCherry on EEs. Yet, it is evident that the regulatory effect of Rab7 on Rab5 is two-sided: Prior to maturation an increased level of GTP-bound Rab7 appear to perturb the binding of Rab5 to EEs. During maturation, however, an increased expression of dominant positive Rab7 seems to facilitate Rab5 coat detachment. Furthermore, we here describe a new maturation dynamic combining the Rab5/Rab7 conversion with a conceivably specific and concentrated vesicular interaction during maturation. The Rab5/Rab7 transition may not exclusively be driven by Rab5/Rab7 on-off kinetics, as incoming Rab7 positive endosomes seems to share their coat at specific microdomains, either through fusion or coat transition.

This study is based on a system where we enlarge endocytic structures utilizing the fusogenic capacity of three different molecules. Based on previous published work in Bakkelab (Stang and Bakke 1997; Gregers, Fleckenstein et al. 2003; Gregers, Nordeng et al. 2003; Landsverk, Barois et al. 2011; Skjeldal, Strunze et al. 2012) and the results presented in this paper we find the Ii-chain induced vesicles to be an important tool for studying endosomal maturation. We believe this system represents a strong tool in the future for discovering important players in the endosomal maturation process.

6. Future Perspectives

The coat dynamics on the separate organelles in the endo-lysosomal pathway are essential for their functions. To be fully able to investigate how the dynamics of these coat proteins interact with each other as well as with proteins found on other organelles is vital for the understanding of how trafficking is regulated. With confocal microscopy, ILEVs and fluorescent techniques we have shown that this is possible to do for Rab5 positive EEs both prior and during maturation, under the effect of overexpressed Rab7-wt and Rab7 mutants.

Even though we feel the experiments show that enlarged vesicles are a useful tool in the study of coat dynamics, there are further experiments that can be conducted to establish a more solid fundament for the continues use of these systems. One of these further studies that could be performed is to examine the dynamics between EEA1 and Rab7 in the Rab5-wt system and the Ii-induced system. We see no point in utilizing the Rab5-Q79L system following the experiments performed here. Investigating the dynamics of EEA1 and Rab7 in the two systems mentioned might be able to shed some light on additional differences between these, and if the overexpression of Rab5, that will only be present in the Rab5-wt-induced system, have any effect on the maturation dynamics.

By using this method there is possible to explore the effect of several coat proteins, such as VPS34, PIKfyve, CORVET, HOPS, Mon1-Ccz1, and their effect on the dynamics of Rab5-wt-mCherry. Together, these studies can reveal a great deal about endosomal maturation. The mapping of the interplay between these proteins can also possibly be used to investigate the dynamics, not only of Rab5, but for Rab7 as well. Employing the same concept to FRAP experiments is also likely to tell us something about the dynamics of coat proteins found on EE prior to maturation, LE posterior to maturation and maybe as well lysosomes during their reorganization from hybrid organelles.

Nonetheless, there is a considerable amount of questions that remains to be answered. We believe that using enlarged vesicles as a tool in the further studies of the endo-lysosomal pathway, combined with live cell imaging techniques, will make a significant difference towards the understanding of maturation.

7. References

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8. Supplementary

Movie 1
The concomitant loss of ctEEA1-GFP and Rab5-wt-mCherry in an Ii-induced cell.

50 Frames. 30 seconds interval.

Movie 2
Rab5-wt-mCherry detachment during maturation in a cell induced with Rab5-wt.

50 Frames. 30 seconds interval.

Movie 3
Rab5-wt-mCherry detachment during maturation in a cell induced with Rab5-Q79L.

50 Frames. 30 seconds interval.

Movie 4
Rab5-wt-mCherry detachment during maturation in a cell induced with Ii.

50 Frames. 30 seconds interval.

Movie 5
Rab5-wt-mCherry detachment and the subsequent Rab7-wt-EGFP attachment during maturation in a cell induced with Rab5-wt.

50 Frames. 30 seconds interval.

Movie 6
Rab5-wt-mCherry detachment and the subsequent Rab7-wt-EGFP attachment during maturation in a cell induced with Rab5-Q79L.

50 Frames. 30 seconds interval.

Movie 7
Rab5-wt-mCherry detachment and the subsequent Rab7-wt-EGFP attachment during maturation in a cell induced with Ii.

50 Frames. 30 seconds interval

Movie 8
Rab5-wt-mCherry detachment and the subsequent Rab7-Q67L-EGFP attachment during maturation in a cell induced with Rab5-wt.

50 Frames. 30 seconds interval

Movie 9
Rab5-wt-mCherry detachment and the subsequent Rab7-Q67L-EGFP attachment during maturation in a cell induced with Rab5-Q67L.

50 Frames. 30 seconds interval

Movie 10
Rab5-wt-mCherry detachment and the subsequent Rab7-Q67L-EGFP attachment during maturation in a cell induced with Ii.

50 Frames. 30 seconds interval

Movie 11
An Ii-induced cell co-transfected with Rab5-wt-mCherry and Rab7-T22N-EGFP. A single endosome which loses its Rab5-wt-mCherry coat, just to regain it is observed.

50 Frames. 30 seconds interval

Movie 12
Rab5-wt-mCherry detachment during maturation in a cell co-transfected with Rab5-wt-mCherry and Rab7-T22N-EGFP. Induced with Rab5-Q79L.

50 Frames. 30 seconds interval

Movie 13
Rab5-wt-mCherry detachment during maturation in a cell co-transfected with Rab5-wt-mCherry and Rab7-T22N-EGFP. Induced with Rab5-wt.

50 Frames. 30 seconds interval

Movie 14
A cell that has been co-transfected with EEA1-RFP and Rab7-wt-EGFP. 2 maturations is observed. Indication of incoming Rab7 positive vesicles binding to Rab7 positive domains during maturation.